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The Role of

bFGF, IGF-I, PDGF and TGF-ß in the Expression of

the Osteogenic Phenotype in

Human Marrow-Derived Bone-Like Cells In Culture

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1. Introduction

Of all structures in the human body which retain the capacity for growth and regeneration throughout post-foetal life, bone tissue possesses an additional potential for continuous internal remodelling and adaptation. To understand these complex physiological processes has been the drive behind research aimed at developing clinically effective methods of promoting repair of bony defects, especially in orthopaedic and plastic surgery. Although millions of fractures occur annually, and the majority heal satisfactorily, 5% to 10% result in delayed union or non-union. It is therefore a matter of ongoing importance to supplement and extend current management and prevention of these problems. It will be the purpose of this paper to present an *in vitro* tissue model, and to address the significance of bone tissue research, its foundations and applications. Broadly, we have based the following inquiry on the same four "cornerstones" upon which all preceding research has been built. We assume:

a) the dependence of bone healing on certain physiological proteins and growth factors,
b) produced by bone cells themselves;
c) the structure and relevance of a pre-clinical *in vitro* bone-culture study, and
d) the potential of practical treatment possibilities evolving in the field of tissue grafting, bone graft substitutes and bone tissue engineering [1].

Purpose of the Study:

Specifically, we considered the effects of four physiological proteins on the growth of bone tissue *in vitro*: transforming growth factor beta (TGF-ß), insulin-like growth factor I (IGF-I), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), and compare these effects to an untreated control group. In what way, if any, do these proteins improve or inhibit bone growth and metabolism as indicated by objectively measured parameters?

Extensive research in this field has already demonstrated that all of these factors can and do affect bone growth in an animal model, both *in vitro* and *in vivo*. A major area, largely unexplored however, is the characterization of these effects on human bone tissue. Using the same experimental parameters as those published in the literature, the goal of this project was to describe the patterns of growth and/or growth inhibition of each of these compounds on human bone cells. These can be summarized as follows:

IGF-I is a stimulator of the osteoblastic phenotype, a temporal regulator of development and is capable of increasing cell survival in human mesenchymal stromal cells.

PDGF functions as an 'early-response' factor; it stimulates osteoprogenitor cells in human bone tissue to proliferate, but may not promote the differentiation to mature osteoblast

bFGF does not significantly stimulate the osteoblastic phenotype, but may hold osteo-progenitor cells in a "stem-state" for a protracted period.

TGF-ß exhibits a biphasic regulation of osteoblast development involving initial suppression of matrix formation and later relative stimulation of cell aggregates into mineralised nodules.

1.1. Bone Development and Growth Factors

Despite its complex makeup of osteoblastic and osteoclastic cell lineages set in a matrix of collagen and non-collagen proteins, bone tissue maintains a *dynamic* state. Studies as early as 1969, most notably by Harris and Heaney[4] demonstrated the skeleton's ability to regulate its own volume, shape and strength in response to external stimulation [5,6]. Chronic mechanical stress, disuse or disease states can alter the balance between osteoclastic resorption and osteoblastic generation for bone catabolism and anabolism respectively, according to the body's current needs. In general, two mechanisms have been suggested for the maintenance of bone volume: 1) systemic regulation by calcium- and phosphate-regulating hormones, e.g. parathyroid hormone, vitamin D, calcitonin, insulin; and 2) local regulation via protein growth factors. Growth factors are proteins synthesized by osteoblasts and non-osteoblastic skeletal and marrow cells. They are believed to act as *autocrine* (osteoblast-derived) and *paracrine* (non-osteoblast-derived) regulators of osteoblast proliferation and matrix biosynthetic activity .[7-10]

Research within the last 15 years has increasingly supported the thesis that growth factors (insulin-like growth factor, transforming growth factor ß, basic fibroblast growth factor, platelet-derived growth factor and bone morphogenetic proteins) are stored in the matrix and osteoid of skeletal tissue [11-13], and are released by the resorptive actions of osteoclasts. Baylink and Finkelman (1993)[5] set up an evolving model to illustrate the local effects of growth factors on bone development (Fig.1-1 and 1-2).

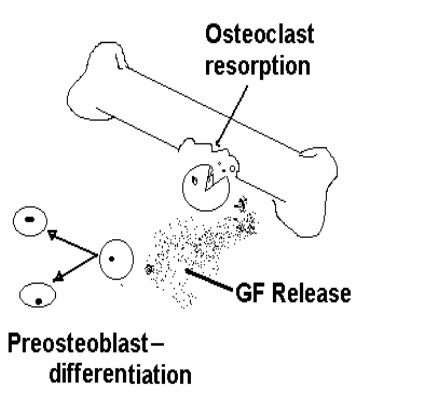


Fig 1-1 Effects of growth factors on bone development-I: Growth factors released from osteoblast are either stored, or released to influence producer osteoblast and other cells. Taken from *Baylink and Finkelman* (1993) [5]

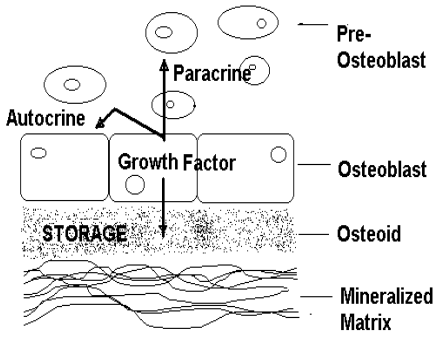


Fig 1-2 Effects of growth factors on bone development- II: Osteoclast resorption releases growth factors (GF) which stimulate preosteoblasts. Taken from *Baylink and Finkelman* (1993) [5]

According to this representation, growth factors are fixed for a time into the bone matrix by means of binding proteins specific for each of the respective factors. In the course of normal physiological utilization of bone calcium reservoir, osteoclastic resorption releases growth factors in a bioactive form to act on osteoblasts and pre-osteoblasts to induce a site-specific replacement of tissue lost to resorption. In this manner, formation and resorption are "coupled" to one another such that they are "*proportional to one another*, *site specific,* and *mediated by an increase in the number of osteoblasts"* [14-17].
This model can be demonstrated *in vivo* by the systemic treatment of animals with agents that stimulate bone resorption[18]. Paradoxically, the animals exhibit not only an increase in the amount of bone tissue resorbed, but also an increase in bone formation; this represents a counter-regulatory mechanism to maintain bone volume at acceptable levels [14,16,19].

1.2. Mesenchymal Stem Cells and Osteoblastic Development

In order to more completely understand the effects of growth factors, it is necessary to look briefly at the origins and developmental stages of osteogenic cells, specifically osteoblasts. The formation and repair of bone tissue begins in the marrow, and involves undifferentiated cellular components that have been partly isolated and identified. It should be mentioned at the outset, however, that much research needs to be done before the precise nature of osteogenesis can be elucidated.

Bone marrow consists of haematopoietic, endothelial and stromal elements, whose network of cells and matrix supply the necessary physical and chemical framework for new bone formation. The stromal cell population can be further subdivided into its individual components: fibroblasts, reticulocytes, muscle cells, adipocytes and osteogenic cells, also known as *mesenchymal cells* [24,25]. These cell lines are believed to originate from a common progenitor, called *(skeletal) mesenchymal stem cell* (MSC) [20-22,26-28], which are defined as the connective tissue elements providing structural and functional support for haematopoesis[26]. Cells of this group are undifferentiated and thought to possess fibroblastic, adipogenic, chondrogenic or osteogenic potential; however, the precise mechanisms that determine the subsequent course of development have not been established in every case. In general, osteoclasts seem to be derived from macrophages and monocytes of the hemopoeitic system[32,33], while osteoblasts stem from the stromal system[30,31]; pioneer work by Friedenstein[29] and Owen[23] was refined by Long (1995)[30], who isolated and characterized human bone precursor cells

from nonadherent marrow cells. According to this study, isolated bone precursors are of three types: osteoprogenitor cells, preosteoblasts and osteoblast-like cells. Each of these subpopulations responds differently to exterior stimuli, osteogenic or otherwise, and so it becomes possible to „steer" the maturation of MSC's toward a given cell lineage (osteogenesis, chondrogenesis, adipocyte etc). Immunologically separated or embryonal pluripotent cell lines can be used to determine not only the *physiological factors* that commit an undifferentiated cell, but also the *optimal time* at which these factors must be present . Wang (1993)[34], for example, induced embryonal mouse mesenchymal cells to differentiate into not only osteoblasts but also chondrocytes and adipocytes.

A crucial question, then, in the field of *in vitro* bone research remains: What are the exogenous determinants of progenitor cell commitment? A model set up by Reddi (1995) illustrates the stages from precursor to osteocyte (Fig.1-3) [35].

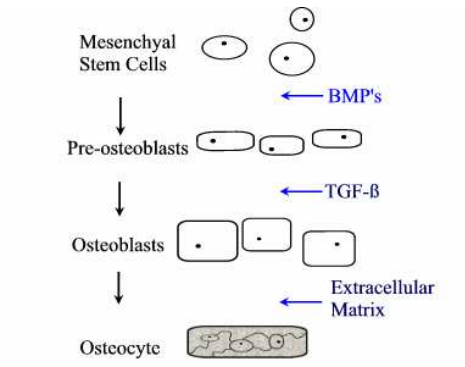


Fig. 1-3 Regulation of Bone Lineage by TGF-ß: Stages of cell development from precursor to osteocyte. Inducible precursor cells (MSC's) become preosteoblasts and finally bone cells. Taken from *Reddi* (1995) [35].

Based on the extensive study currently being done on growth factors and bone morphogenetic proteins (BMP's), Reddi proposed that certain of these proteins are stage-specific for MSC's commitment to a specific pathway: BMP's, for example are the primordial signal for the initial commitment of undifferentiated mesenchymal stem cells (also called *inducible osteogenic precursor cells)* to differentiated preosteoblasts (*determined osteogenic precursor cells*). The two subsequent steps, from preosteoblasts to osteoblasts and from osteoblasts to osteocytes are mediated by e.g. TGF-ß, and components of the extracellular matrix, respectively, leading to the formation of new bone [35]. *Inducible* osteogenic precursor cells, i.e. MSC's, require a molecular signal for initiating the differentiation process, while *determined* cells i.e. post-preosteoblasts, will begin differentiating into bone even without an exogenous signal [35,36].

1.3. TGFß Superfamily and Bone Morphogenetic Proteins

1.3.1 Cell Differentiation: Role of Bone Morphogenetic Proteins (BMPs)

Any discussion of osteogenesis must include a short look at the role of bone morphogenic proteins and their effects on MSC and elsewhere. Even though no BMP's

were used in this project, they are mentioned for two reasons: 1) their importance to the regulation of bone lineage, and 2) the fact that information on BMP's may help explain the characteristic effects of other growth factors, for example TGF-ß.

The term BMP was first coined by Marshall Urist, MD, who in 1965 first reported extraskeletal osteoinduction [13], and has come to refer to any substance that can induce ectopic bone formation in a standard *in vivo* rodent assay [37]. Roughly, BMPs are potent members of the multigene TGF-ß family; as little as 50 ng of purified extract is sufficient for *in vivo* activity [42]. Within the marrow microenvironment, BMPs induce multi-potential stromal cells to differentiate along the osteoblastic pathway [2,38-40] (as summarized in Fig. 1-3) and in doing so, blocks the development of MSC along other cell lineages, thereby functioning as negative regulator as well [21]. Murray *et al* (1993) demonstrated for example that BMP will inhibit myotubule formation in committed C2C12 myoblasts in a concentration-dependent manner [41], and Gimble *et al* (1995) demonstrated the ability of BMP-2 to inhibit adipogenesis in mutipotential stromal cells [40]. Gimble's model illustrating the actions and relationship of BMPs to the mesodermal cell lineages is summarized in Fig. 1-4.

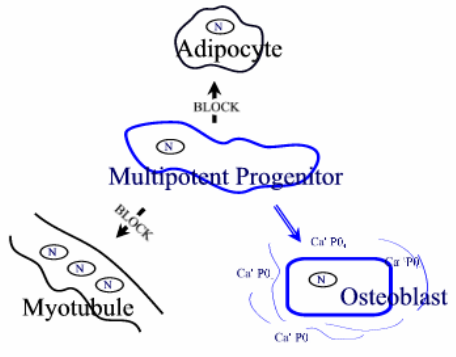


Fig. 1-4 Model of BMP regulation of mutipotential stromal cells along the mesodermal lineages. Taken from *Gimble et al* (1995) [40].

These considerations become important in view of the effects of TGF-ß on bone cell culture, discussed in Section 4.4.

1.3.2 Cell Differentiation: Role of TGF-ß

Having established the role of BMPs in the early commitment of *inducible* stromal cells, the next stage to be examined is the further differentiation of pre-osteoblasts once they are *determined.* As indicated in Fig. 1-3, the polypeptide TGF-ß, one of the most abundant of the known regulatory factors stored within the matrix [12], seems to be responsible for the stimulation of preosteoblast proliferation; this would serve to either create a large pool of committed cells, or alternatively to increase further differentiation of the osteoblasts themselves and thereby secretion of mineralising matrix proteins [11,47]. Preliminary findings concerning the *in vitro* effects of TGF-ß have been variable: in some human osteoblast-like cells (osteoblastic osteosarcoma cells), TGF-ß has stimulated differentiated function and inhibited proliferation [48], while other researchers have seen stimulation of both differentiation and proliferation [165] in the same type of cells. This

discrepancy may depend on the presence or absence of serum in *in vitro* cultures; TGF-ß is stimulatory in the absence [39] but inhibitory in the presence [60] of serum. The proliferative effect of TGF-ß also appears to be dependant on maturity of the cells, (i.e. *more* proliferative on cells at an *intermediate* stage of bone development, *less* proliferative in cell cultures derived from mature organisms), and on concentration in culture (i.e. the stimulatory effect of TGF-ß on DNA synthesis decreases at high TGF-ß concentrations) [189].

However, it seems clear that in populations of primary mammalian stem cells (in most studies taken from foetal rat calvaria), TGF-ß

1) inhibits MSC differentiation to osteoblasts, while
2) stimulating proliferation of the osteoblasts themselves [47,49,50].

In other words, the major effects of TGF-ß on cell growth and differentiation are restricted to the proliferative phase of the culture, *before* osteoblasts express a mature phenotype. This leads to an initial suppression of "mature bone cell" characteristics, such as calcium deposition and nodule formation [50]. However, continued exposure to TGF-ß leads cells to exhibit a second, positive growth response in the form of increased matrix formation and mineralization, i.e. a *biphasic* effect. These findings are further supported by studies implicating TGF-ß in the inhibition of osteoclast activity, i.e. osteoclasts are inhibited by TGF-ß from resorbing newly-formed ossic tissue [58,59].

With regard to these inhibitory effects, accumulating evidence suggests that TGF-ß works closely in co-operation with 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). This steroid hormone plays an important role in both calcium homeostasis and skeletal metabolism, and induces the transcription of major functional osteoblast products, such as osteocalcin and osteopontin. $1,25(OH)_2D_3$ acts by coupling to distinct Vitamin D Response Elements (VDREs) located on the genetic promoter for osteocalcin synthesis; TGB-ß inhibition of osteoblast differentiation involves the selective down-regulation of Vitamin D interactions with osteocalcin VDREs [132,133]. TFG-ß directly downregulates the expression of the osteocalcin gene in normal osteoblastic and osteosarcoma cells in rats [159].

1.3.3 TGF-ß and Osteoblast Recruitment

Bone development occurs in a sequential cascade consisting of three steps: chemotaxis, mitosis and differentiation [35,50]. Previous studies have suggested that an initial step in the coupling process during bone formation is likely to be a biochemical attraction of osteoblast-like cells to the site of previous resorption [51], which, interestingly, is another of TGF-ß's principle functions and has been demonstrated in rat and human osteoblasts; maximal stimulation of chemotaxis was observed at concentrations as low as 5-15 pg/mL [51,166]. Lind *et al* (1997) [166] studied the concentration-dependent stimulation of osteoblastic migration in response to TGF-ß, and noticed a decline in chemotactic response at higher doses. This phenomenon, also noticed in cells of the immunological system, seems to permit the pull of an osteogenic cell type from some distance away; migration is stopped when the cells are in the proper position and the higher

concentration of TGF-ß induces metabolic functions instead of migration [52,53]. Lind formulates a hypothetical mechanism by which TGF-ß might exert its chemotactic influence: TGF-ß is released in an inactive form, bound to a binding protein to protect against initial hydrolytic cleavage. After release as an active protein, TGF-ß inhibits osteoclasts and enhances osteoblastic recruitment [54]. Osteoblasts then migrate toward the resorption lacunae, where they are stimulated by TGF-ß together with other growth factors to proliferate and produce matrix proteins [55,166]. This increase in matrix proteins is the second part of TGF-ß's *biphasic* influence; the variation, however, in culture model, dose ranges, delivery methods, protein isoform and end points for analysis make it difficult to establish these mechanisms *in vitro* as anything more than a rough guideline of TGF-ß´s possible effects.

Structurally, TGF-ß belongs to a growing family of polypeptide factors, among them the activins, inhibins and BMPs, which share certain physical and functional characteristics. TGF-α is an unrelated peptide exhibiting a higher degree of homology with epidermal growth factor (EGF) than with the TGF superfamily. Molecules producing TGF-ß have been observed in serum, blood platelets, chondrocytes and fibroblasts as well as extraskeletal locations such as placenta, kidney and astrocytes [56,57]. There are an additional five isoforms of TGF-ß designated numerically TGF-ß1 through TGF-ß5, differing in receptor affinity and bioactive potency.

1.4. Growth Factors: bFGF, PDGF, and IGF

The distinction between the following growth factors and the TGF-ß superfamily is based for the most part on genetic composition; all such bioactive molecules exhibit overlapping functions and effects on the growth of skeletal tissue, and interact to varying degrees with one another within a physiological system. Thus the following description of the growth factors involved in this study will focus on their individual characteristics, keeping in mind their mutual influence on each other.

1.4.1 Basic Fibroblast Growth Factor (bFGF)

It is perhaps reasonable to discuss basic fibroblast growth factor immediately following TGF-ß because of the regulatory relationship between them; a study by Noda *et al* (1989) [61] reported an enhanced expression of TGF-ß genes in osteoblasts treated with bFGF. This discovery has been borne out in recent years in studies focussing on mesenchymal growth factors, which include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and bFGF. Fibroblast growth factors are a group of polypeptides originally isolated from brain and pituitary extracts, and make up what is known as the heparin-binding growth factor (HBGF) family. Currently there are nine members which have been isolated in mammals; all are structurally related and are designated FGF-1 to FGF-9 [66] . The following investigation deals only with a prototypic member, basic fibroblast growth factor (=FGF-2) because of its importance as a modulator of cartilage and bone growth and differentiation [26].

Previous *in vitro* studies have ascribed various functions to bFGF, among them the stimulation of chondrocytes [67,68], osteoblasts [69,70], angiogenesis [78] and in regulation of

hematopoeisis [71]. Both inducible marrow stromal cells and osteoblasts produce bFGF [62], and since bone matrix has been shown to contain this growth factor in abundance [12], it has been suggested that bFGF may preferentially trapped in the bone matrix with specific binding proteins in addition to being synthesized by bone cells. Canalis *et al* (1988) [70] determined the primary function of bFGF as being *mitogenic*; specifically, it reduces average cell replication time by shortening the G1-Phase of the mitotic cycle. These observations indicated that bFGF *enhances osteogenesis* by stimulating bone cell *replication*, which increases the number of collagen-synthesizing cells, but has a directly *inhibitory* effect on osteoblastic type-I collagen synthesis [70]. In a study with periostium-derived cells, all osteoblastic parameters i.e. osteocalcin and collagen gene expression, alkaline phosphatase (AP) activity and calcium content were decreased, whereas overall DNA content was enhanced. Iwasaki *et al* (1995) showed that bFGF is also a potent inhibitor of differentiation: MSCs in periostium were prevented from differentiating into either chondroblasts or osteoblasts, but were themselves induced to replicate [72]. Especially important for the coupling between bone formation and resorption is consequently the inhibition by bFGF of osteoclast formation [62]. These initial results indicate that in mesenchymal bone cell cultures, bFGF serves as a mitogenic factor for both differentiated and undifferentiated cells, but an inhibitor of cell lineage determination .

In stromal marrow culture, however, several studies have noted that bFGF enhances proliferation *and* osteogenic expression of human marrow stromal cells [3,69,138]. Treatment of culture with bFGF and glucocorticoids by Pri-Chen *et al* (1998) resulted in a four-fold *increase* in osteocalcin in addition to an overall DNA increase [134], which may reflect either bFGF stimulation of an expanded proportion of stromal osteogenic cells, or direct activation of the osteocalcin promoter gene. Martin *et al* (1997) established the tendency of bFGF to maintain MSC in a particular functional state, e.g. a *stem state,* and therefore to support growth and expansion of osteogenic precursors [138]. These data are supported by *in vivo* studies that demonstrate increased levels of bFGF in developing (proliferating) chick limb buds; these levels then decline when differentiation begins [147]. Taken together, these investigations suggest that bFGF may play a dual role in regulating osteogenic potential by:

1) stimulating proliferation of committed and uncommitted *progenitors* in marrow MSC by maintenance in an embryological responsive state (stem state). The osteogenic potential of MSC is *highly increased* in the presence of bFGF, making conceivable the performance of autogenic bone reconstruction without the need for large amounts of bone marrow aspirate [138];

2) reducing differentiation but increasing proliferation of *periosteal* (i.e. already committed) bone cells, which is necessary in the event of bone growth or fracture healing [72].

Both of these functions are greatly dependant on dose and duration of treatment.

With regard to interactions with TGF-ß as mentioned above, bFGF has exhibited in several studies its ability to either counteract the effects of TGF-ß by downregulating the

number of TGF receptors in target cells [73], or to enhance TGF-ß effects by stimulating its gene expression in osteoblasts. This second responsibility would perhaps serve as a negative feedback mechanism against the inhibitory effects of bFGF.

1.4.2 Platelet-Derived Growth Factor (PDGF)

Early studies with platelet adhesion to damaged vascular intima demonstrated a significant local increase in smooth muscle cell and fibroblast proliferation, leading researchers to search for the source of this growth-promoting activity. The knowledge that cell-free, plasma-derived serum did not exhibit this activity, and that it could be restored by the reinfusion of platelets eventually led to the isolation of Platelet-Derived Growth Factor by Ross *et al* (1974)[110]. PDGF, like its counterparts, initiates a variety of biological responses in mesenchymal stem cells, which include proliferation, chemotaxis, and increased synthesis and degradation of extracellular matrix; it is especially expressed during osseous wound healing, is mitogenic and chemotactic for osteoblastic cells *in vitro*, and stimulates new bone formation *in vivo* [74-76]. The effects of PDGF are in some ways quite similar to those of bFGF; Canalis *et al* (1989) demonstrated that PDGF is located in the matrix, stimulates DNA synthesis in rat marrow cultures and exhibits a strong mitogenic effect in, but is not specific for, osteoblast-like cells. In addition, PDGF may either *inhibit* the osteoblastic phenotype by decreasing AP activity, collagen synthesis and matrix apposition rates, or have *no effect*, for example on osteocalcin production. These effects could also be mediated in part by PDGFs inhibition of skeletal IGF, the most prevalent local stimulator of the differentiated function of osteoblasts [80]. Generally, PDGF tends to increase replication but not differentiation [79]. This thesis is supported by Yu *et al* (1997) who found that continuous PDGF treatment increased histone expression, indicative of enhanced proliferation (replication) but suppressed differentiation as expressed by a decline in alkaline phosphatase, collagen and osteocalcin in rat calvarial cells [186]. In contrast, Pfeilschifter *et al* (1992) reported the *increase* in collagen synthesis by 50% and a decrease in AP activity by 20% following treatment of rat calvarial cells with PDGF, suggesting that PDGF may have varying production patterns and effects *in vivo* depending on the developmental stage of cells affected [55], i.e. a strong dependence on *temporal* factors. In contrast to bFGF, however, PDGF *stimulates* bone resorption by significantly increasing osteoclast number [81] and collagen degradation, possibly due to an elevation in collagenase levels [77,164]. Consistent with the above characteristics is the finding that PDGF stimulates A- and B-chain mRNA production, an effect which is not specific to bones but also occurs in vascular endothelium [82].

Structurally, PDGF exists as the product of two genes that encode two distinct chains, PDGF-A and PDGF-B. These chains share 60% amino acid homology and can combine to form a covalently-linked homo- or heterodimer comprising either A or B subunits i.e. PDFG-AA, -BB, or AB. This study employs one of these three isoforms, PDGF-BB, which predominates in circulation and is the most potent *in vitro* at equivalent doses [74,79]; PDGF-BB, but not –AA, stimulates bone resorption and interstitial collagenase expression in osteoblasts [63]. These effects seem to be dependent on the affinity of PDGF-BB for PDGF-receptor types α and β while PDGF-AA occupies only the α-receptor. In fact, most, but not all, differences in isoform potency are due to different binding affinities for receptor subtypes [74].

PDGF synthesis takes place in stromal cells, macrophages and of course in platelets, and is stored in matrix; Rydziel *et al* (1996) [63] demonstrated PDGF-BB transcripts in normal osteoblasts, identifying it as a systemic and local regulator of bone cell function.

1.4.3 Insulin-Like Growth Factor (IGF)

Insulin-like growth factor, also known as somatomedin C (Sm-C), is a growth-hormone (GH)-dependent polypeptide synthesized primary in visceral organs, neural tissue and skeletal cells. It acts as both a systemic and local modulator of skeletal growth, and systemic agents; particularly parathyroid hormone and steroid hormones like estradiol have been shown to regulate the production and secretion of IGF in cells of the osteoblast lineage [80,87,88]. Human serum contains several IGFs: the two major forms IGF-I and IGF-II plus several minor-sequence variants which comprise less than 10% of total physiological insulin-like activity in the human body. IGF-I and –II are single-chain polypeptides consisting of four domains, the first two of which (A and B) bear up to 43% sequence homology with the A and B domains of human proinsulin. Sequence homology between IGF-I and –II is 62%, but their effects and their respective active concentrations differ on several points. We chose to study IGF-I because of the high doses of IGF-II necessary to cause a relatively small stimulation of bone matrix production [187]. The following discussion will therefore focus exclusively on the subject of this particular study, IGF-I.

In terms of its effects, IGF-I operates in an autocrine/paracrine manner, regulating the proliferative and differentiative functions of bone cells [5,86]. Unlike bFGF and PDGF described previously, IGF-I has been shown to stimulate proliferation *and* matrix synthesis *in vitro*, i.e. it increases the replication of cells of the osteoblastic lineage (probably preosteoblasts) and enhances osteoblastic collagen synthesis and matrix apposition rates and thereby the expression of proteins like alkaline phosphatase and osteocalcin [55,89] . This effect seems to be due to at least two regulatory signals: firstly, its direct influence on differentiated osteoblasts (enhancement of type I collagen production) and secondly, an increase in osteoprogenitor cell replication, giving a larger number of functional osteoblasts; these effects can be dissociated from each other biochemically, suggesting independent mechanisms [90]. Insulin, by comparison, also stimulates collagen synthesis and matrix production but does not alter cell replication [91]. Like many other local factors, IGF-I acts on both bone formation and resorption: first Mochizuki (1992) and more recently Hill *et al* (1995) [92,93] demonstrated that IGF-I stimulates bone resorption *in vitro* by enhancing both osteoclast formation (hemopoeitic recruitment) and activity. Interestingly, cells of the osteoblastic lineage mediated these effects; osteoclasts isolated from rat long bones did not respond to IGF-I if incubated alone. Only following the addition of osteoblastic derivatives of human osteosarcoma cells did the osteoclasts respond, suggesting that osteoblasts release a soluble factor to stimulate bone resorption [92,94] as outlined in Section 1.1.

The question concerning IGF-I's effects as a catabolic or anabolic agent becomes more significant in light of the *in vivo* action of IGF-I on mature, as opposed to growing, bone. IGF-I delivered by osmotic pump to osteoporotic rats *increased* bone formation rate and trabecular number; no such effect was noted in animals that did not have osteoporosis

[184]. Generally, current research shows IGF-I to stimulate mRNA expression of AP, procollagen, osteocalcin and osteopontin, but its *in vitro* effects on stromal cells appear to be age-related; older adult animals are more significantly affected than younger adults[185].

1.5. *In Vitro* Cell Culture and Experimental Parameters

Up to this point, we have been using information regarding the actions of certain physiological growth factors obtained, for the most part, through the cultivation and examination of tissue cultures in an artificial environment, i.e. the long-term *in vitro* growth and maintenance of mammalian bone cells, human and otherwise. Tissue culture represents the main experimental *ex vivo* methodology by which researchers have established the osteochondral potential of MSC in bone marrow; subsequent stages leading toward animal (and eventually human) *in vivo* trials have typically involved bone and cartilage development in rat diffusion chambers, i.e. porous tricalcium phosphate-hydroxyapatite ceramic cubes containing marrow-derived cells, implanted subcutaneously into syngeneic or immunocompromised hosts [100,101]. An important question that must therefore be addressed is whether or not cell cultures can be utilized as accurate representation of a mammalian system, and by extension: Can the data extrapolated from an *in vitro* experiment be applied to an animal and eventually a human model?

In vitro systems cannot entirely imitate an *in situ* microenvironment, and yet they provide useful models with which to study some, but not all aspects of osteoblast function. Techniques and conditions that isolate MSC in culture have been developed for avian [102], rodent [103] and canine [104] models, leading to the extensive cultivation, subcultivation and characterization of human stromal cells [105-107]. Essentially, *in vitro* experiments in which stromal cells are induced to form bone fall into two broad categories: one in which the culture medium is supplemented with a phosphate donor (beta glycerophosphate, ß-GP) and/or a steroid (dexamethasone, dex), and a second in which the medium is supplemented with an „inductive" peptide purified from demineralized bone matrix (DBM), for example one of the bone morphogenic proteins [108].

Bone cells, as described previously, undergo a consistent *in vitro* developmental sequence (proliferation, matrix maturation and mineralisation) whose parameters can be regulated by the administration of bioactive signals [85]. In addition, the temporal expression of bone matrix proteins during *de novo* bone formation *in vivo* has revealed distinct patterns for individual proteins [109,110]. These same patterns of expression are seen during bone formation *in vitro*, allowing inferences to be made between the appearances of certain proteins, and e.g. cell differentiation stage [111,112]. Such protein level parameters have become the most accurate available measure of osteoblastic function in research with bone cultures, as osteoblasts consistently exhibit a series of characteristics that have proven useful in their identification: alkaline phosphatase activity, increased intracellular cAMP in response to parathyroid hormone, the ability to form nodules with a mineralised extra cellular matrix comprised of type I collagen, and increased levels of the bone-specific proteins osteopontin, osteonectin, bone sialoprotein (BSP) and bone Gla protein (osteocalcin) [106]. While all can be used as valid parameters

to (indirectly) quantify the presence of osteoblasts in culture, only a select few, for reasons outlined below, were employed in the present study.

1.5.1 Bone Marker Proteins

Osteocalcin, the most abundant non-collagenous protein (about 15%), is one of the most reliable markers of bone tissue [113,114], and growing data indicates that it is the most specific to date for the osteoblast phenotype [120]. It contains three residues of the calcium-binding amino acid (-carboxyglutamic acid (GLA), synthesized by a vitamin K-dependent carboxylation of specific residues in a peptide chain; some authors consider it to be unique to osteoblasts / odontoblasts or tumour cells with osteoblastic potential [126,128]. Although originally thought to be involved in mineralization, the generation by Ducy *et al* (1996) [116] of mice lacking an osteocalcin gene showed that osteocalcin is a negative regulator of bone formation: the so-called 'osteocalcin-knockout mice' exhibited increased cortical thickness of the long bones but no concomitant increase in the number of osteoblasts. Interestingly, the content and rate of apposition of bone minerals were identical between wild-type and mutant mice, suggesting that osteocalcin functions by limiting bone matrix resorption without affecting mineralisation [115,116]. Osteocalcin, along with bone sialoprotein, is expressed *after* osteoblasts have differentiated (Fig.1-8) [112]; its production has been identified with the committed step that includes matrix mineralisation.

Collagen is the single most abundant animal protein in mammals, accounting for about 30% of all proteins. Thirteen different types have been so far isolated in humans, designated types I-XIII; for the purposes of bone research, the most appropriate have proven to be types I and II. Collagen type I is not specific for bone but is also found abundantly in skin, tendon, ligament and cornea, where it comprises 80-90% of total collagen [118], nevertheless it is highly expressed by cells of connective tissue and the osteoblastic lineage, i.e. by the perichondrium, periosteum and osteoblasts [117,119]. Significantly, Collagen I is directly associated with mineralization of bone nodules (described below), although some authors have not found a correlation between biochemical and histological determination of matrix apposition [55]. Temporally, it is expressed during the period of cellular differentiation and matrix deposition (see Fig.1-8), and is synthesised as *procollagen,* a larger precursor molecule. Procollagen consists of mature collagen with extension peptides, which are cleaved from the collagen molecule by specific proteases prior to incorporation into a growing collagen fibril. The release of these peptides provides a stoichiometric representation of the production of collagen.

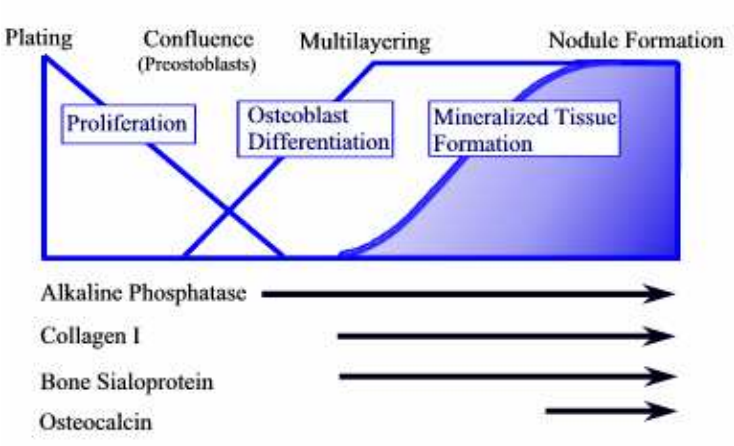


Fig. 1-5 . Taken from Li *et al.* (1996) [112], the above diagram depicts the stages of osteoblast differentiation in fetal rat calvarial cells *in vitro.* Human marrow-derived culture and osteosarcoma cells exhibit similar characteristics [145,146] .

1.5.2 Non-Protein Parameters:

Bone nodules

One of the most intriguing parameters of *in vitro* osteogenesis is the formation of colonies of differentiated osteoblasts and their associated matrix, called 'bone nodules'. These nodules comprise a multilayered system with an uppermost layer of cuboidal osteoblastic cells capable of producing an osteoid matrix similar to woven bone [120,121]. Immediately beneath this 'osteoblast layer' is a seam of unmineralised matrix which can be seen with an electron microscope to contain collagen fibrils and exhibit the histological characteristics of osteoid. In addition, the matrix of mineralised nodules has been demonstrated to contain many of the components found in bone: collagen type I, III and V, osteonectin, osteocalcin and osteopontin. Based on these morphological and biochemical similarities, many researchers regard bone nodule structure as being very close to that of embryonic/woven bone synthesized *in vivo* [123-125].

According to extensive work in this area by Bellows *et al* [122] the formation of nodules appears to be dependant on three distinct factors: the ability of cells to multilayer *in vitro*, the presence of ascorbic acid, and the addition of ß-glycerophosphate (ß-GP) to the culture medium. The *ability to multilayer* is important for the simple reason that cells respond to 'contact inhibition' if layering is not possible, and will not expand into a 3D structure when so hindered. *Ascorbic acid* seems to stimulate the formation and hydroxylation of collagen, allowing for the sufficient deposition of collagen to create a localized elevation in the culture surface. Finally, the mineralisation process requires an

organic phosphate donor, and *ß-GP*, a substrate for AP, may contribute at least in part by providing this source of phosphate, even though it is not the same organic phosphate form found *in vivo* [126,127].

Having briefly discussed the general conditions necessary to induce nodule development, the next phase involves an examination of the four successive morphological steps of bone nodule formation, elucidated by Nefussi *et al* (1997)[183]:

cell proliferation with formation of multicellular layers – AP and non-collagen proteins (NCP) such as osteocalcin and osteopontin are *not* expressed until the creation of a three-dimensional microenvironment is completed; this may even include a transient cell-*de*differentiation state, to speed up proliferation during the first 24 hrs after plating.

cell surface morphological changes with cell differentiation – the production of NCPs begins after 3D cell organization.

cell activity with matrix formation and maturation – the cells, i.e. osteoblasts below the nodule surface, begin to actively synthesize matrix.

woven bone matrix mineralisation – with formation of active bone surface and mature osteocytes. The mineralisation of human MSC in culture is associated with an increase in calcium deposition in the extracellular matrix, especially calcium phosphate [120,135].

These stages are not successive, but take place concomitantly at different locations during nodule development.

The Role of Glucocorticoids in Bone Nodule Formation:

It is important to note that a number of chemical elements are indispensable additions to medium if bone nodules are to develop at all: glucocorticoids have been shown to be a prerequisite for the expression of osteogenic markers by stromal bone marrow cells derived from both animals and humans [134,135], possibly because of their ability to help recruit progenitors to the osteogenic lineage [134,136]. Interestingly, some preliminary findings indicated that glucocorticoid, which induces the formation of nodules in animal bone-cell cultures, actually *decreases* nodule formation in human bone-derived cell cultures [123]. Furthermore, Cheng *et al* (1994) [135] noted that unlike rodent and bovine marrow cultures, human MSC cultures in the presence of glucocorticoids *only* (no growth factors added) are unable to form any nodules at all, which may be partially explained by the tendency of glucocorticoid to induce the *differentiation* (recruitment) of osteoprogenitor cells while inhibiting their *proliferation*. This would also account for the well-documented inclination of long-term pharmacological doses of glucocorticoids to cause osteopenia *in vivo*: the inability of osteoprogenitor cells to properly multiply, as may occur during an intense regime of high-dose glucocorticoids, may lead to relatively diminished osteoblast numbers and bone loss [135,144]. However it seems clear that for marrow cultures, physiological concentrations of glucocorticoids are necessary for both the differentiation of osteoprogenitor cells into cells that exhibit the osteoblastic phenotype, as well as mineralisation of the matrix synthesized by these cells. In addition,

glucocorticoids significantly affect bone-cell adhesion factors and the attachment of osteogenic cells to the extracellular matrix [137].

Taken together, this information suggests that, on one hand, the *in vitro* induction of nodule formation in either bone-cell or bone marrow culture represents a valid and workable model for the study and understanding of *in vivo* bone characteristics. On the other hand, however, the dissimilar biochemical responses of animal versus human culture in the presence of glucocorticoids suggests that nodule-, and perhaps ultimately bone-formation and -mineralisation occur in two related yet comparable ways; future growth factor research must include the more intense characterization of human cell populations.

1.5.3 Serum Elements Necessary for Culture Growth

Osteogenic Supplements

One final element of nodule culture is the widely practised augmentation of culture medium with two Osteogenic Supplements (OS): ß-glycerophosphate (ß-GP) and ascorbic acid (AsA).

ß-Glycerophosphate (ß-GP): Even though the signals necessary for MSC to differentiate into various cell types are still not entirely understood, it has been proposed that ß-GP, in conjunction with AP, plays a significant role in promotion of both osteogenic differentiation and mineralisation *in vitro*, resulting in bone-like tissue formation [108,120,139,140]. ß-GP also appears to raise inorganic phosphate (P*i*) and depress AP levels in cell cultures [141], whereby concentration seems to be critical: Becerra *et al* (1996) [108] reports that only concentrations below 2mM enhance physiological mineralization, whereas higher levels (up to 10mM) additionally stimulate increased calcium deposition.

Ascorbic Acid: AsA functions as a cofactor in the hydroxylation of proline and lysine residues in collagen [142,] as well as increasing the synthesis of non-collagenous matrix proteins [143]; it is generally considered as essential additive to osteogenic cell cultures [107].

Proper Medium Selection

The medium constituents play an important role in the eventual growth pattern and behaviour of cell cultures, especially marrow-derived MSC. Cell seeding density, type of tissue-culture plastic and source of foetal calf serum are known to affect the development potential of cultured cells [100,107,142]. Of special significance is the addition of foetal bovine serum, which contains many of the factors essential for *in vitro* proliferation: hormones; substrate-attachment molecules; binding proteins for the transport, presentation and utilization of essential molecules; and finally, nutrients which may be absent from synthetic medium or present in only insufficient concentrations. The following experiment makes use of a chemically defined medium (IMDM), augmentated with Horse Serum (HS) and Fetal Calf Serum (FCS) to permit attachment and proliferation of primary culture [24].

1.6. Experimental Proposal

The following experiment was established to investigate the effects, whether positive or negative, of TGF-ß, IGF-I, bFGF and PDGF on osteoprogenitor cells. To do this, we set up an *in vitro* model using marrow stromal cells aspirated from the pelvic bones of adult human subjects. These cells were allowed to grow in an artificial, incubated environment for 7 days until a confluent layer of cells was visible, and were then continuously exposed to a single one of each the growth factors at a given concentration over a period of 24 days. To investigate dose dependency, two concentrations of each growth factor were used, 1 ng/mL and 10 ng/mL. A control group of cell culture from the same subject was grown under the same experimental conditions but without exposure to any growth factor.

In order to measure the effect of these growth factors, we maintained a record of changes in parameters associated with maturation of *in vitro* bone cells as described in the literature. Visible cell morphology, cell count and matrix calcification (quantified by staining) were taken as indicators of culture activity and proliferation. Biochemical analysis for the bone proteins osteocalcin and procollagen, as well as development of bone nodules, were taken as indirect evidence of cell differentiation to the osteoblastic phenotype.

An important part of the study was the observation of growth factor behaviour over an established *time course*; while many studies test growth factor activity as a statistical function of *dose-dependence*, very few authors, if any, show temporal passage. The experiment was planned so that all cultures in a given group were taken from the same subject (see Sec. 2.3), and enough sets were incubated at the beginning to allow for each to be analysed once and then discarded.

The results thus acquired were analysed, interpreted and compared to findings reported by other research groups.

2. Materials and Methods

2.1 Cells and Culture Conditions

Human mesenchymal stem cells (hMSC) were obtained from normal human donors (ages 20-30 years), via approximately 30-40 mL iliac crest aspiration. Isolation of MSC populations took place within 3-4 hours of operation; cells were separated from hematologic constituents of marrow aspirates by mixing with methyl cellulose (5mL) and Medium (~25mL) in centrifuge tubes (Corning 50 mL) followed by removal and centrifugation of MSC at 700 x *g* and 4°C for 10 min.
Supernatant pellet was resuspended in 10 mL medium; cell count to determine plating density was done with a 0,0025 mm^2 hemacytometer (Neubauer, Germany). Cells were plated to a density not exceeding $1x10^6$ cells/cm^2 on 4-well SonicSeal slide wells (Nunc, Illinois) and 24-well flat-bottom plates (Corning, MA).
Plated cells, grown to confluence for 7 days before start of experiment, were kept incubated at 37° in a humidified atmosphere (Heraeus Instruments, Germany) consisting of 95% air and 5% CO_2. Thereafter, culture medium was changed every 3 days for a total of 24 days.

Medium:
Iscove's Modified Dulbecco's Medium (IMDM) was supplemented with antibiotics (penicillin and streptomycin 1000 IU/mL), Foetal Calf Serum (10%), Horse Serum (10%), 0.1 mL hydrocortisone stock and osteogenic supplements (10mM ß-glycerophosphate, ascorbic acid 50 g/mL)

2.2. Organization of Groups

The present investigation concerns itself with the effect of growth factors on human marrow-derived MSC. The growth factors, as outlined in Sections 1.2 and 1.3 were divided up according to the following schema:

GROUP 1 Marrow aspirate (35mL) from male patient, 22 yrs old.

GROUP	*FACTOR 1*	*FACTOR 2*	
1a	IGF-1 1ng/mL	PDGF 1ng/mL	
1b	IGF-1 10ng/mL	PDGF 10ng/mL	Control

Tab. 2-1

GROUP 2 Marrow aspirate (35mL) from female patient, 28 yrs old.

GROUP	*FACTOR 3*	*FACTOR 4*	
2a	bFGF 1ng/mL	TGF-ß 1ng/mL	
2b	bFGF 10ng/mL	TGF-ß 10ng/mL	Control

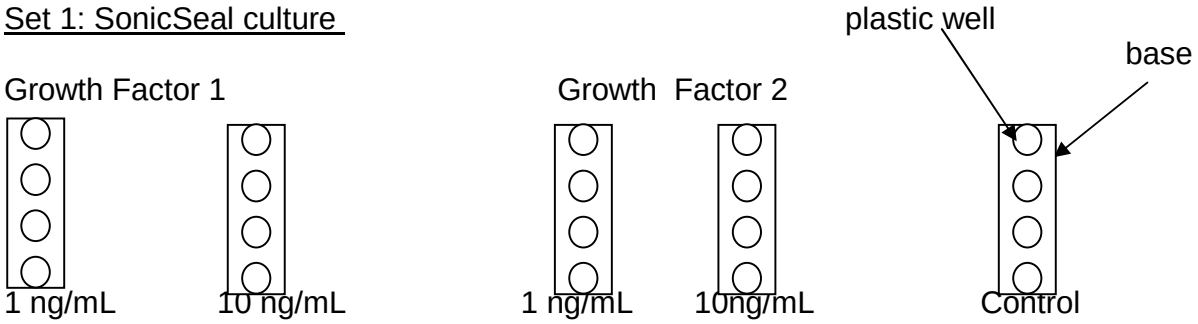
Tab. 2-2

Each group was cultured in duplicate, i.e. one set cultured in SonicSeal 4-well plates, and one set cultured in Corning 24-well flat-bottom plates (see Fig 2-1). The SonicSeal plates, which have a detachable plate and are therefore better suited for microscopic

examination, were used for cell morphology analysis, while the Corning plates were used for calcium staining procedures.

Group *n*

Set 1: SonicSeal culture



Set 2: Corning 24-well culture

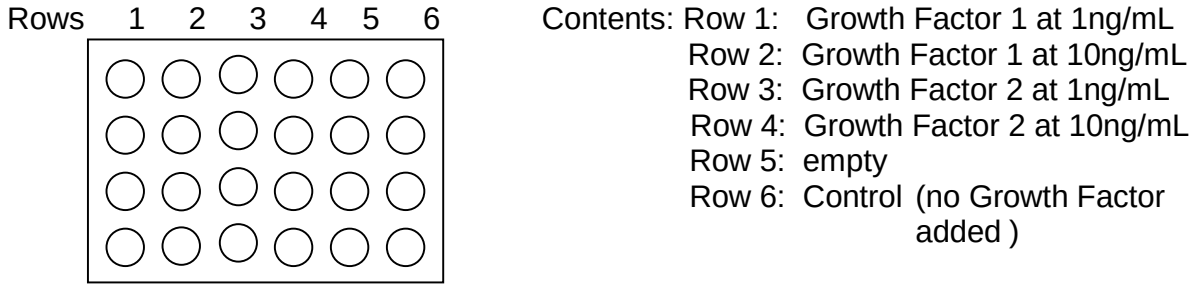


Fig. 2-1 Organization of Groups. Each growth factor was added to 2 sets of culture (Sets 1 and 2) for duplicate measurements. Factors 3 and 4 were cultured in the same manner.

After plating, cultures were left for one week to grow to confluence. The first day was then designated Day 7, marking the start of the 31-day experimental period. Medium was changed and photographic records made every three days **i.e. Days 7, 10, 13, 16, 19, 22, 25, 28 and 31**, allowing assessment of biochemical and morphological changes occurring within each 72-hr period.

2.3. Measurement of Parameters Characteristic of the Osteoblastic Phenotype*:*

2.3.1 Physical Parameters

Cell Morphology

Cells present in bone marrow culture, especially those such as fibroblasts and adipocytes visible at low light-microscope magnifications, assume identifiable patterns during their passage through various stages of growth. Individual cells were described as being spindle-shaped, cuboidal, polygonal (multifaceted) or filamentous, while patterns of aggregate cells were seen as whorls (i.e. broad spiral or whirled formation), islet-formation (i.e. isolated islands of cells without definitive borders but with no contiguous abridgement to any other cell groups) or nodular (i.e. cell aggregates with a raised three-

dimensional structure, or with a definite perimeter separating it from the surface layer). Furthermore, to characterize the spectrum of overall covering of the culture plate, cell layers were noted for their sparse, intermediate or confluent growth over the three-week period.

Documentation of cell morphology was done only in the SonicSeal cultures, using phase-contrast micrographs (Leitz, Germany) using Kodak Ektachrome 64T Colour Reversal Film, at magnifications of 1.2x, 4x, 10x, 20x and 32x.

Cell Count

Cell count was done with a hemacytometer (0.0025 mm², Neubauer, Germany) and light microscope (Leitz Diavert 40-100x magnification, Wetzlar, Germany). It reflects the absolute number of non-adherent cells free in medium. On every assessment day, (i.e. **days 7,13,19, 25 and 31**), 6mL from each group was set aside as a sample, centrifuged at 3 000 U/min and the supernatant removed. The pellet was resuspended in 1mL medium and stained in Tuerk solution at a ratio of 50µL medium to 50µL Tuerk's, after which 10µL was removed to the haemocytometer for counting. In the event that the cell concentration of a given day was not sufficiently high to allow adequate distinction between the groups, the ratio of medium to stain was altered to 90:10, respectively, and calculations made to accommodate this change.

Bone nodules

As discussed in Sec.1.4.2., bone nodule formation represents a measure of the bone culture's ability to form calcified structures similar to woven bone. For quantification of nodules, cell layers were stained *in situ* using the von Kossa technique (see Section 2.4.2.), and nodules counted by placing plates under a light microscope at 4x magnification. Only those stained black by the von Kossa procedure were enumerated.

Formation of Adipocyte-Like Cells

As discussed in Sec 1.2 *Mesenchymal Stem Cells*, the pluripotent population of marrow precursors is thought to contain progenitors common to other cellular components of the body, among them adipocytes (Fig. 1-5). Given the assumption that our growth factors stimulate the osteoblastic phenotype, we observed the cultures for the degree to which other types of cell populations were either promoted or suppressed. The term 'adipocyte-like' is used here to indicate that these cells were not characterized beyond their morphological appearance.

2.3.2 Staining Procedures

von Kossa Staining

For assessment of mineralization potential of bone cell culture, the plates were first drained of medium, then fixed with Histochoice Tissue Fixative (Sigma, MO) for 20 min. The cells were then washed with deionised water and exposed to 5% silver nitrate solution and UV light for 15 min. Following rinsing (de-ionised water) and addition of 1% Pyrogal solution, residual silver nitrate was neutralized with 5% thiosulphate. Plates were assessed for the presence of stained areas, representing calcified nodules and surface area. An overall rating, assigned to each plate on **days 7,13,19, 25 and 31** was based on an estimate of the percent surface area covered by stain (**A**). We assessed *only* those wells in which at least 75% of the well bottom was covered by a cell layer. Further, each well was assigned a grade for depth of colour exhibited (**B**). Although there was some variability, a number of growth patterns were recognizable based on the following ranking:

A= Percentage of layer stained:	**B**= Depth of colour
Score 0= no stain observed	Score 0= stained area is transparent
1= 1-29% of surface stained	1= stained area translucent, no colour
2= 30-59% of surface stained	2= translucent with opacity in **<** 50% of stained surface area
3= 60-90% of surface stained	3= translucent with opacity in **>** 50% of stained surface area

Table 2-3 Quantification of percentage of layer stained and depth of colour using objective score gradient.

A final ranking was then given by plotting the score of percent layer stained **(A)** (Table 2-3) and assigning a colour code to each histograph bar to signify depth of stain **(B)** (Table 2-4). This total score was then graphed to visualize *both* simultaneously. Results are shown in Section 3.3.

Stain Intensity:	Score Attained in Table2-3 (**B**)	Colour Code
	0……	
	1……	
	2……	
	3……	

Table 2-4: Quantification of depth of colour observed after staining using a colour gradient

2.3.3. Biochemical Parameters

Osteocalcin (Gla-OC)

Median osteocalcin levels were measured by solid-phase enzyme immunoassay (Gla-Type EIA-Kit, Lot No. 004, TaKaRa, Japan) according to the manufacturer's instructions. This assays utilizes a set of mouse monoclonal antibodies (Mabs) to detect osteocalcin in both its carboxylated and decarboxylated forms. The procedure is a two-step 'sandwich' method in which Gla-OC is bound to immobilized anti-Gla-OC (solid-phase on the bottom of the microtitre-plate well), and then tagged with peroxidase (POD)-anti-OC. A further reaction between POD and a chromogenic solution containing tetramethyl-benzadine (TMB) results in colour development with intensity and absorbance proportional to the amount of Gla-OC present in the sample.
Briefly, the procedure was done by incubating a medium sample (1:4) for 2 hours at room temperature (18-25℃) in a microtitre-plate we ll coated with anti-Gla-OC antibody. After aspirating the reagent from the well, the plate was washed three times, Chromogenic Solution added, and the plate incubated for a further thirty minutes at room temperature on a horizontal shaker set at 700±100 rpm, avoiding direct sunlight. Afterward, Stop-Reagent was pipetted into the well and the absorbance read at 450 and 490 nm.

Type I Collagen

Median levels of collagen in serum were measured using a solid-phase enzyme immunoassay (Prolagen-C® EIA-Kit, No. 8003, Metra Biosystems, California U.S.A) according to the manufacturer's instructions. This assay uses a three-step indicator (incubation with immobilized and free antibody, enzyme conjugate and substrate) to detect the presence of *procollagen,* a larger precursor molecule, as a stoichiometric representation of type I collagen (see Section 1.4).
Briefly, serum samples were first diluted with an assay buffer at 1:12 and incubated in microtitre-plate wells coated with purified murine anti-Collagen-**I** carboxyl propeptide (C**I**CP) at room temperature (18-25℃) for 120 ±5 minutes. After three washings with buffer solution, the wells were incubated with rabbit anti-C**I**CP antibody for 45-50 minutes at room temperature. The molecules were then tagged by incubating them for 45-50 minutes at room temperature with a lyophilised goat anti-rabbit IgG antibody conjugated to alkaline phosphatase. Following a further three washings with buffer, the final incubation with a p-nitrophenyl phasphatase substrate for 30-35 minutes at room temperature was done, and the reaction ended with addition of Stop-Solution. Optical density was read at 405 nm and sample results analysed after correcting for dilution

3. Results

3.1 Physical Parameters

3.1.1. Cell Morphology

All cultures began as non-confluent fibroblastic layers. Partial obstruction by red blood cells during the first few days of the study did not significantly hinder micrographic documentation, as these red blood cells were gradually removed over the course of medium replacement, allowing an unrestricted view of the cell layer.

		Control	TGF-ß 1ng/ml	TGF-ß 10ng/ml	bFGF 1ng/ml	bFGF 10ng/ml	IGF-I 1ng/ml	IGF-I 10ng/ml	PDGF 1ng/ml	PDGF 10ng/ml
6 days of initial culture growth										
Day 7	**1**	n-c	n-c	n-c	n-c	n-c	n-c	n-c	n-c	n-c
	2	s	p	p	p	p	s/p	s/p	s/p/cu	s/p/cu
	3	-	-	-	-	-	-	-	-	-
Day 10	**1**	n-c	n-c	n-c	c	n-c	n-c	n-c	c	c
	2	s	p	p	p/f	p	s/p	s/p	s/p/cu	s/p/cu
	3	-	-	-	-	-	-	-	w	w
Day 13	**1**	c	int	int	c	c	c	c	c	c
	2	s	p/f	p/f	p/f/cu	s/p/cu	s/p/cu	s/p/cu	s/p/cu	s/p/cu
	3	w	isl	isl	w	w	w	w	w	w
Day 16	**1**	c	spar	spar	c	c	c	c	c	c
	2	s/p	p/cu	p	s/p/cu	s/p/cu	s/p/cu	s/p/cu	s/p/cu	s/p/cu
	3	w/nod	isl	isl/nod	w	w	w	w	w	w
Day 19	**1**	c	spar	spar	c	c	c	c	c	c
	2	s/p	p/cu	p/cu	s/p/cu	s/p/cu	s/p	s/p	s/p	s/p
	3	w/nod	isl/nod	isl/nod	w	w	w	w	w/nod	w
Day 22	**1**	c	spar	spar	c	c	c	c	c	c
	2	s/p	p/cu	p/cu	s/p	s/p	s/p	s/p	s/p	s/p
	3	w/nod	isl/nod	isl/nod	w	w	w/nod	w	w/nod	w/nod
Day 25	**1**	int	spar	spar	c	c	c	c	c	c
	2	s/p	p/cu	p/cu	s/p	s/p	s/p/f	s/p/f	s/p	s/p
	3	w/nod	isl/nod	isl/nod	w	w	w	w	w/nod	w/nod
Day 28	**1**	int	spar	spar	c	c	c	c	c	c
	2	s/p	cu	cu	s/p	s/p	s/p/f	s/p/f	s/p	s/p
	3	isl/nod	isl/nod	isl/nod	w	w	w/nod	w/nod	w/nod	w/nod
Day 31	**1**	int	spar	spar	c	c	c	c	c	c
	2	s/p	cu	cu	s/p	s/p	s/p	s/p	s/p	s/p
	3	isl/nod	isl/nod	isl/nod	w	w	w/nod	w/nod	w/nod	w/nod

Tab. 3-1: Influence of TGF-ß, IGF-I, PDGF and bFGF on Cell Morphology. Cell shape and layer development were observed and changes noted. 1)Cells were described as having one of four possible shapes: spindle-form, polygonal (multi-faceted), cuboidal and filamentous. 2) Confluence of the cell layer was described as being confluent or non-confluent, with degrees of confluence: sparse, intermediate or fully confluent (dish surface covered completely). 3) The macroscopic appearance of the cell layer was characterized as having a whorled appearance, islet-form (isolated cell groups), or nodular (raised cell-aggregates).

Day *n* **1**	Line 1 =degree of layer confluence: spar=sparse / int=intermediate / c= fully confluent / n-c=nonconfluent

2 Line 2 =appearance of cells: s=spindle / p=polygonal / cu=cuboidal
f=filamentous

3 Line 3 =appearance of layer: w=whorled / isl=islet-form / nod=nodular

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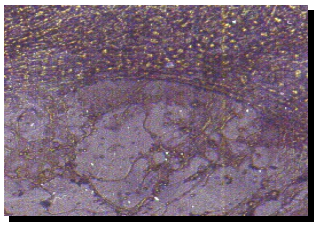
Cell Morphology: Photographic Examples

The following images are examples of the morphological distinctions made during observation of cell development:

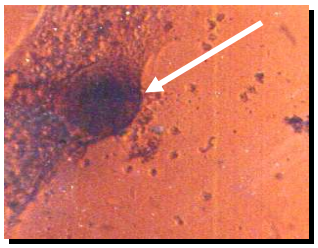
a) Degree of layer confluence:



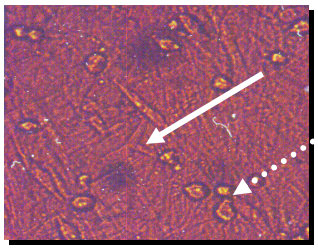
Non-confluent cell layer: the upper half of the photograph shows a solid layer formation, which is either sharply demarcated or branching into areas of the culture plate not yet covered by cells.

Confluent cell layer : this formation was present only after Day 10 in all cultures; variations between the confluent and non-confluent stages were designated "**sparse**" or "**intermediate**".

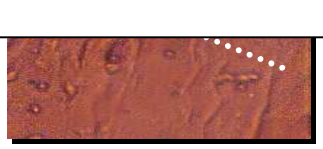
b) Types of Cell Shapes



Nodule: the formation indicated by the white arrow shows a bone nodule situated on a small **cell-islet**. The rest of the image consists of scattered individual cells which are not part of any larger cell layer.



Spindle-shape: as shown by the solid arrow, the majority of matrix cells assumed an elongated form with the central cell nucleus. The dotted arrow indicated a **cuboidal** cell of the type which mainly floated free, not as part of any structure.



lid arrow) were seen as
, primarily, but not exclusively,
xpansion. The dotted arrow
olygonal cells, which could not be classified in any of the previous categories.

3.1.2. Bone Nodule Formation

We observed the presence of nodules (see Section 1.4), which stained positively for calcium, in all groups, including control. Nodule count, time and duration of presentation varied widely between groups, as presented in the following diagrams. Even though an initial 7 days were allowed for culture growth to begin before any nodule count was made, in none of the groups were any nodules observed until Day 19. For each set of results, control value was set at 1,0 in order to permit comparison between the groups:

IGF-I gave variable results depending on concentration (Fig. 3-18). In general, there seemed to be an earlier increase in nodule formation in the 10ng/mL (Day 19) than in the 1 ng/mL group (Day 25). However, the latter group reached a higher count relative to control. By Day 31, both groups had returned to levels close to control.

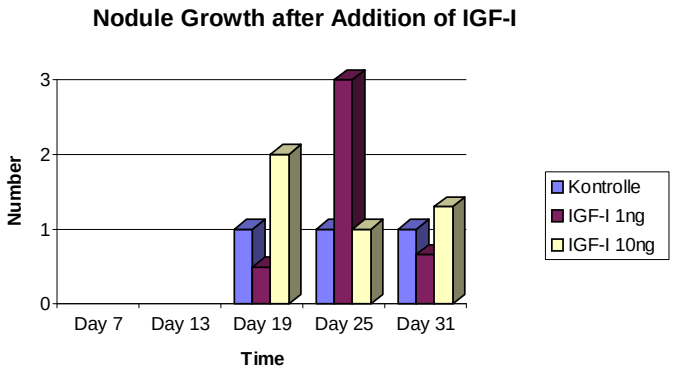


Fig. 3-1. The Influence of IGF-I on Bone Nodule Induction

PDGF nodule count (Fig. 3-19) showed a similar pattern between the two concentrations. Both the 1 and 10ng/mL groups exhibited a slight rise above

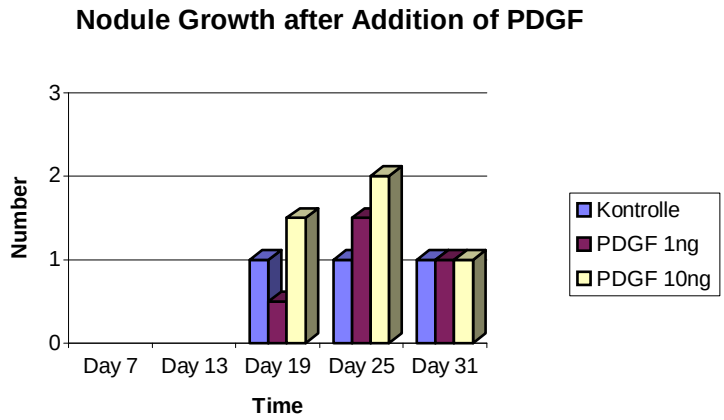


Fig. 3-2 The Influence of PDGF on Bone Nodule Induction

control on Day 25, (with the 10ng/mL group producing a higher count) and had returned to control levels by Day 31.

The **bFGF** plates (Fig.3-20) clearly showed a reduction in the number of nodules counted in comparison to the control group. Not only were nodules in both the 1 and 10ng/mL groups absent until Day 25, but also the absolute count was in both concentrations below that of control.

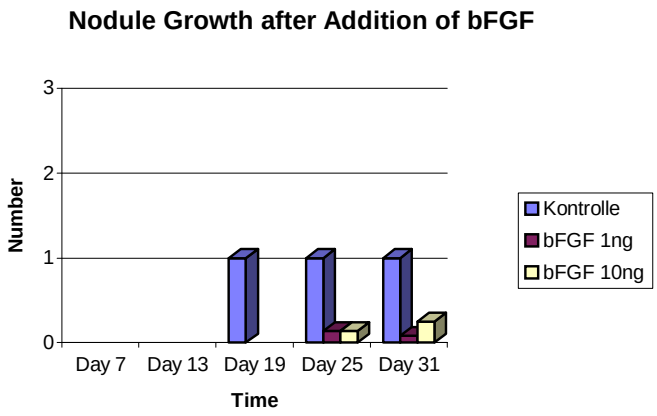


Fig.3-3 The Influence of bFGF on Bone Nodule Induction

For **TGF-ß** (Fig. 3-21)**,** the nodule count showed its greatest rise in the 10ng/mL group on Day 19. Afterwards, it's levels dropped close to that of control until Day 31. The 1ng/mL group also exhibited nodules from Days 19-31, but remained below control for the duration of the study.

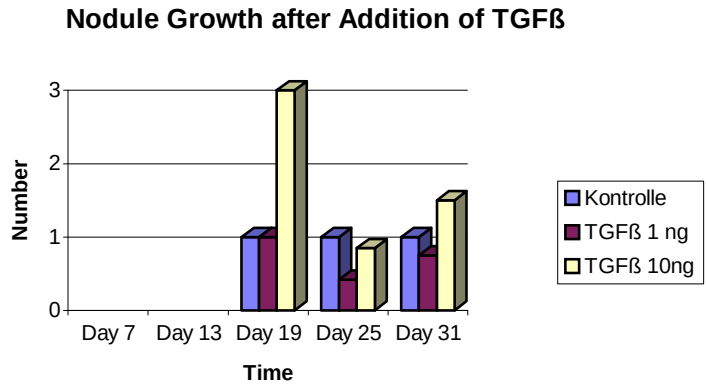


Fig. 3-4 The Influence of TGF-ß on Bone Nodule Induction

3.2 Staining Procedure

3.2.1 Calcium content as quantified with v. Kossa Stain

All cultures stained positively for calcium. Generally, each group reached what appeared to be a 'staining maximum', before and after which colour intensity was noticeably weaker. In addition, there seemed to be a positive correlation in all growth factor groups between the percentage of surface area stained and the intensity of stain uptake. Each plate received a rating (as outlined in Section 2.4.2.), according to the total surface area covered by stain, and according to the depth of stain intensity. The following illustrations (Fig. 3-5, 3-6, 3-7, 3-8, 3-9) are based on the results listed in Tables 3-2 and 3-3, and depict the rating assigned each of the results obtained on **days 7,13,19, 25 and 31.** The *height* of the histogram bar reflects the relative size of the area stained, whereas the *colour* of each bar reflects the intensity of Kossa stain. Fig 3-9 depicts a summation of the relative course of all growth factors as given by layer rating points for each day. For the sake of simplicity, all treatment groups in this diagram are displayed as though compared to a single control; control plates in Groups 1 and 2 both exhibited the same layer rating even though taken from different subjects (see Table 2-1 and 2-2).

	Day 7		Day 13		Day 19		Day 25		Day 31	
	Layer	Colour	Layer	Colour	Layer	Colour	Layer	Colour	Layer	Colour
Control	0	0	1	1	1	1	1	2	1	1
IGF-I 1ng	0	0	1	1	1	2	2	2	1	1
IGF-I 10ng	0	0	1	1	1	2	2	2	1	1
PDGF 1ng	0	0	1	1	3	3	2	2	2	2
PDGF 10ng	0	0	1	1	3	3	2	2	2	2

Tab.3-2: v Kossa Stain Intensity: Quantification of percentage of layer stained and depth of colour using score gradient as outlined in Table 2-3.

	Day 7		Day 13		Day 19		Day 25		Day 31	
	Layer	Colour	Layer	Colour	Layer	Colour	Layer	Colour	Layer	Colour
Control	0	0	1	1	1	1	1	2	1	2
bFGF 1ng	0	0	2	1	2	1	2	1	1	2
bFGF 10ng	0	0	2	2	2	2	2	1	1	1
TGFß 1ng	0	0	0	0	0	0	1	1	1	2
TGFß 10ng	0	0	0	0	0	0	1	2	1	2

Tab. 3-3: v Kossa Stain Intensity: Quantification of percentage of layer stained and depth of colour using score gradient as outlined in Table 2-3.

The **IGF-I** plates exhibited identical results in both concentrations. Until Day 19 less than 30% of the surface stained positive, after which an increase in both surface area and

stain intensity above control was noted. This returned to initial levels by Day 31. The **PDGF** group showed its strongest gain in area and stain intensity on Day 19 (60-90%), considerably stronger than control. PDGF then dropped to between 30- 59% for the remaining measurements. The **bFGF** cultures showed a significant level of surface staining on Day 13; moreover, opaque areas the 10ng/mL group were induced early in the study (Day 13) and then tapered off. Its 1 ng/mL counterpart did not show opacity until Day 31.Finally, **TGF-ß** showed very little tendency to stain, remaining negative until Days 25-31, and even then, only exhibited stain uptake in below 29%. This was inferior to the control group, which had begun to stain by Day 13.

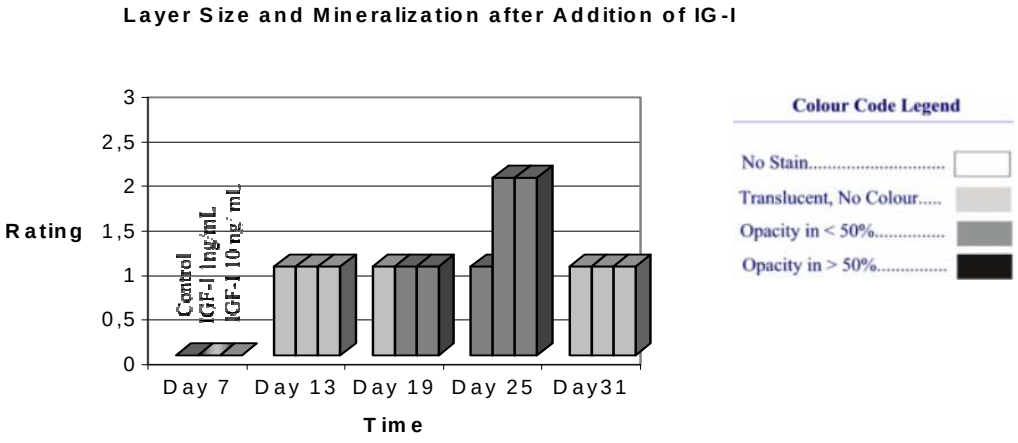


Fig. 3-5: Influence of IGF-I on Culture Mineralization as Quantified by von Kossa Stain Technique. Interpretation of Rating: 0= No stain, 1=1-29% of surface stained; 2= 30-59%, 3=60-90%

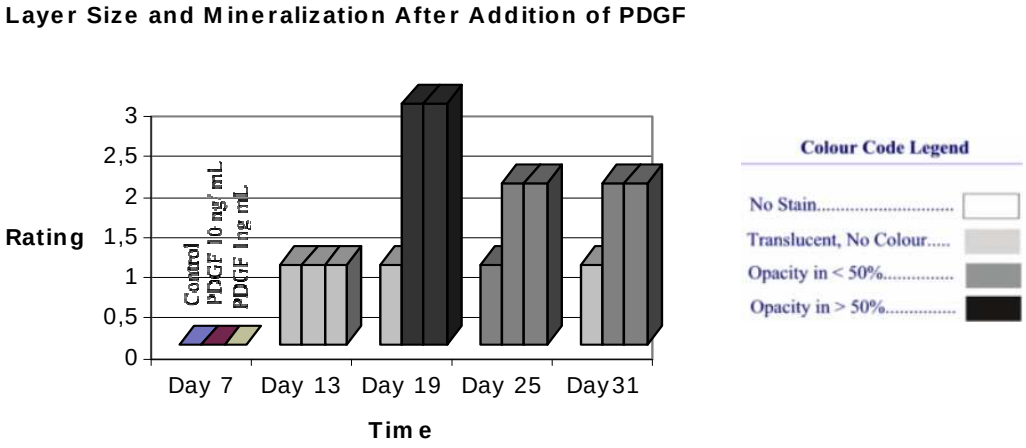


Fig. 3-6: Influence of PDGF on Culture Mineralization as Quantified by von Kossa Stain Technique. Interpretation of Rating: 0= No stain, 1=1-29% of surface stained; 2= 30-59%, 3=60-90%

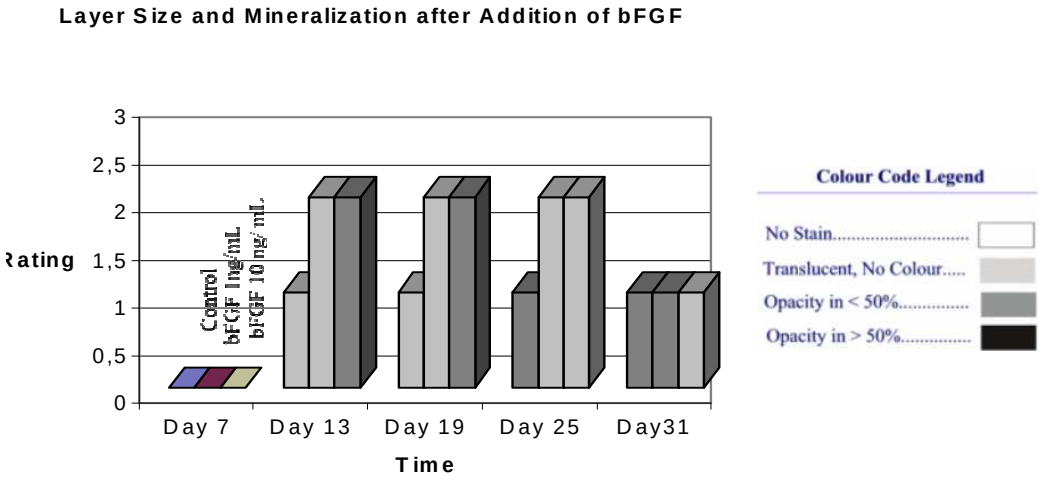


Fig. 3-7: Influence of bFGF on Culture Mineralization as Quantified by von Kossa Stain Technique. Interpretation of Rating: 0= No stain, 1=1-29% of surface stained; 2= 30-59%, 3=60-90%

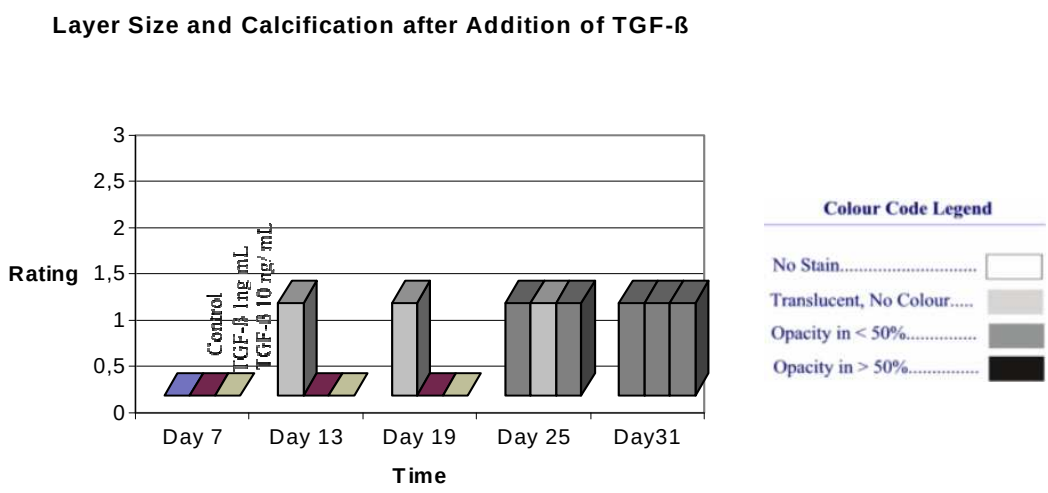


Fig. 3-8: Influence of TGF-ß on Culture Mineralization as Quantified by von Kossa Stain Technique. Interpretation of Rating: 0= No stain, 1=1-29% of surface stained; 2= 30-59%, 3=60-90%

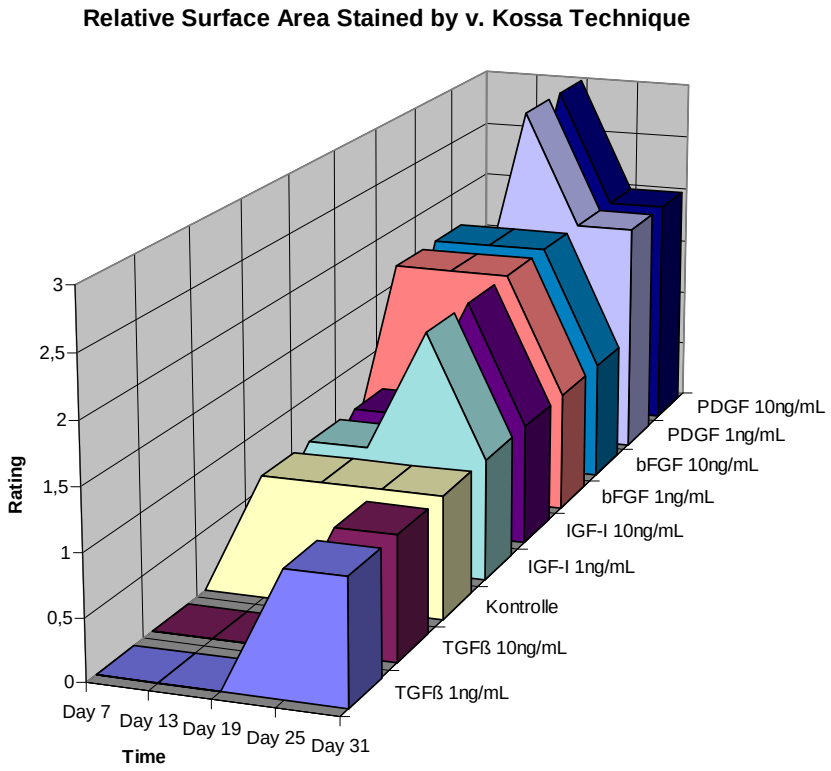


Fig. 3-9 – Summary of relative layer rating between treatment groups based on percent of layer stained by v. Kossa method.

3.2.2 Photographic Record of Calcium Content as Quantified with v. Kossa Stain

The Effect of IGF 1µg On the Quantity of Calcium Content in *In Vitro* Cultures, Made Visible By von Kossa Staining Technique and Photographed With an hp Photosmart 715 Digital Camera. Slow progression with peak calcification by Day 25.

IGF 1µg
Day 7

IGF 1µg
Day 13

IGF 1µg
Day 19

IGF 1µg
Day 25

IGF 1µg
Day 31

The Effect of IGF 10µg On the Quantity of Calcium Content in *In Vitro* Cultures, Made Visible By von Kossa Staining Technique and Photographed With an hp Photosmart 715 Digital Camera. Slow progression with peak calcification between Days 19- 25.

IGF 10 µg
Day 7

IGF 10µg
Day 13

IGF 10µg
Day 19

IGF 10µg
Day 25

IGF 10µg
Day 31

The Effect of PDGF 1µg On the Quantity of Calcium Content in *In Vitro* Cultures, Made Visible By von Kossa Staining Technique and Photographed With an hp Photosmart 715 Digital Camera. Accelerated calcification with peak production approximately Day 19.

PDGF 1µg
Day 7

PDGF 1µg
Day 13

PDGF 1µg
Day 19

PDGF 1µg
Day 25

PDGF 1µg
Day 31

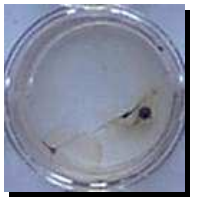
The Effect of PDGF 10µg On the Quantity of Calcium Content in *In Vitro* Cultures, Made Visible By von Kossa Staining Technique and Photographed With an hp Photosmart 715 Digital Camera. Accelerated calcification with peak production approximately Day 19.

PDGF 10µg
Day 7

PDGF 10µg
Day 13

PDGF 10µg
Day 19

PDGF 10µg
Day 25

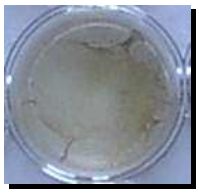


PDGF 10µg
Day 31

The Effect of bFGF 1µg On the Quantity of Calcium Content in *In Vitro* Cultures, Made Visible By von Kossa Staining Technique and Photographed With an hp Photosmart 715 Digital Camera. Rapid calcification with plateau production after Day 13.

bFGF 1µg Day 7

bFGF 1µg Day 13



bFGF 1µg Day 19

bFGF 1µg Day 25

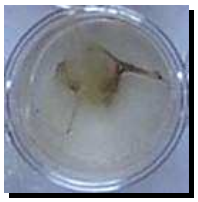
bFGF 1µg Day 31

The Effect of bFGF 10µg On the Quantity of Calcium Content in *In Vitro* Cultures, Made Visible By von Kossa Staining Technique and Photographed With an hp Photosmart 715 Digital Camera. Rapid calcification with plateau production after Day 13.

bFGF 10µg Day 7

bFGF 10 µg Day 13

bFGF 10 µg Day 19

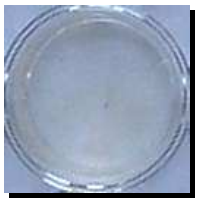


bFGF 10 µg Day 25

bFGF 10 µg Day 31

The Effect of TGF-ß 1µg On the Quantity of Calcium Content in *In Vitro* Cultures, Made Visible By von Kossa Staining Technique and Photographed With an hp Photosmart 715 Digital Camera. Gradual calcification with maximum production after Day 25.

TGFß 1µg Day 7



TGFß 1µg Day 13

TGFß 1µg Day 19

TGFß 1µg Day 25

TGFß 1µg Day 31

The Effect of TGF-ß 10 µg On the Quantity of Calcium Content in *In Vitro* Cultures, Made Visible By von Kossa Staining Technique and Photographed With an hp Photosmart 715 Digital Camera. Gradual calcification with maximum production after Day 25.

TGFß 10µg
Day 7

TGFß 10µg
Day 13

TGFß 10µg
Day 19

TGFß 10µg
Day 25

TGFß 10µg
Day 31

3.3 Biochemical Parameters.

3.3.1. Osteocalcin

The enzyme immunoassay method of detecting osteocalcin, described in 2.4.1., allowed us to follow the progression of osteocalcin production in each of the cultures. In one set of results, the absolute levels are given; in the second, the control value is set at 100% in order to allow comparison between the experimental sets.

IGF-I induced osteocalcin levels which were, for the most part, below those of the control group. The 10ng/mL group in particular ran in a pattern that was almost diametrically opposed to that of the control, showing a "second peak " spike at Days 13-19. This peak was also visible in the 1ng/mL group when graphed as a percent of the control value (Fig.3-10); in absolute values, this group showed a stable pattern with a slight rising tendency.

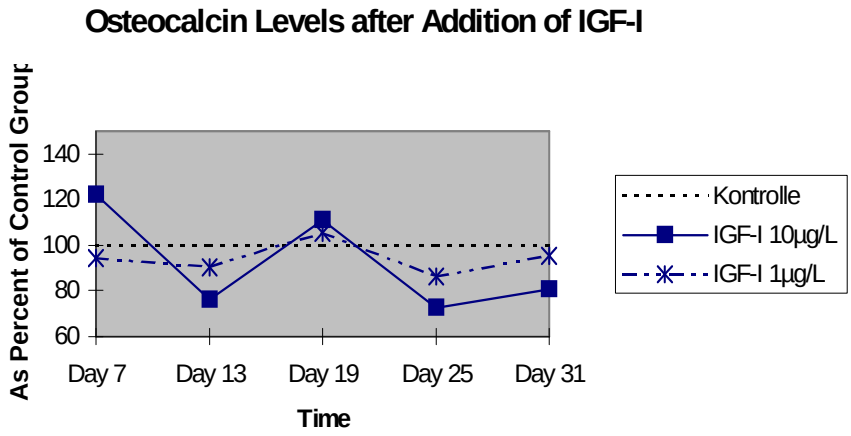


Fig. 3-10: Influence of IGF-I on Osteocalcin Levels at 1ng/mL and at 10 ng/mL: Relative Values (control=100%)

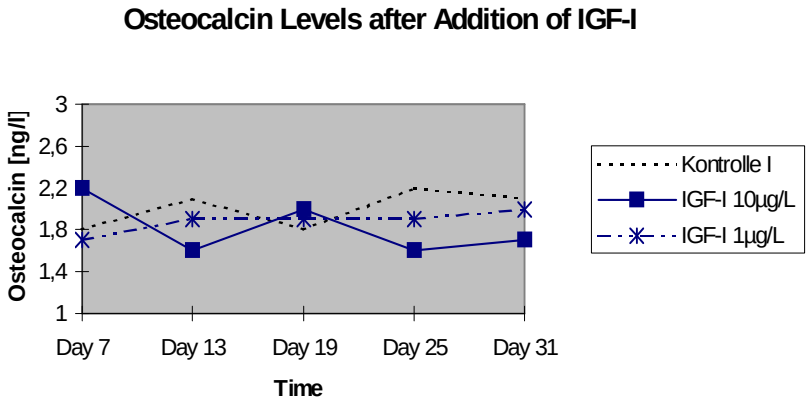


Fig. 3-11 : Influence of IGF-I on Osteocalcin Levels at 1ng/mL and at 10 ng/mL : Absolute Values

PDGF showed a distinct rise in both in both concentrations above the control group (Days 13-19), whereby the 1ng/mL group reached a higher absolute value (Fig. 3-13). After Days 19-25, OC levels began sinking to levels similar to control until the end of the study.

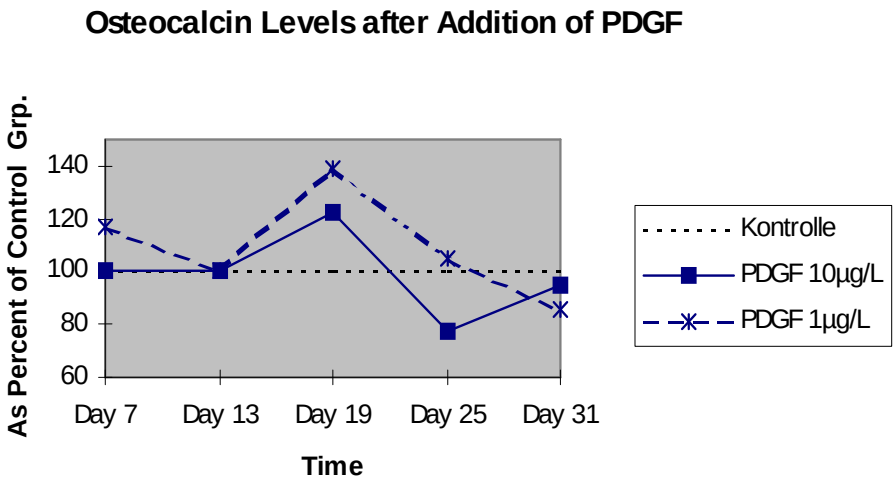


Fig. 3-12: Influence of PDGFon Osteocalcin Levels at 1ng/mL and at 10 ng/mL: Relative Values (control=100%)

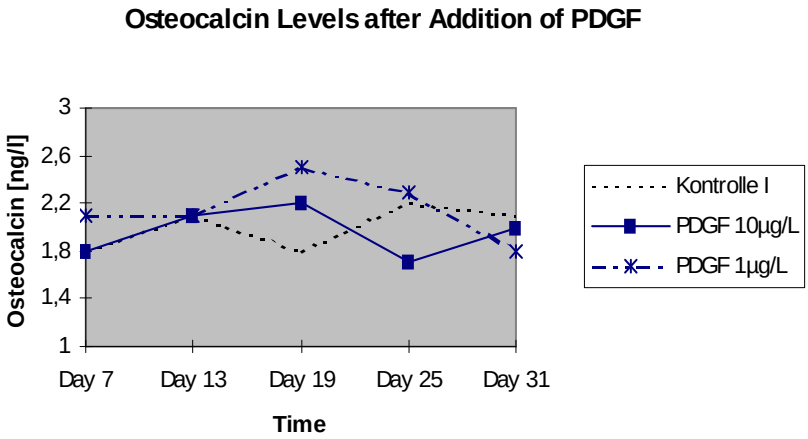


Fig. 3-13 Influence of PDGFon Osteocalcin Levels at 1ng/mL and at 10 ng/mL: Absolute Values

bFGF exhibited a sharp rise in the 1ng/mL group, which sank after Day 13 and thereafter remained in the vicinity of the control values. The 10ng/mL group rose more slowly above control, reaching its highest peak on Day 25 and then rapidly falling to control levels (Fig. 3-14 and 3-15).

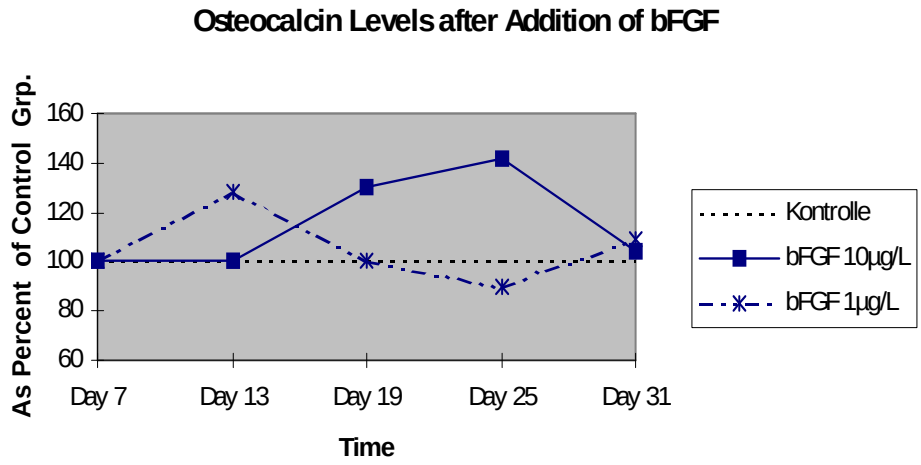


Fig. 3-14: Influence of bFGF on Osteocalcin Levels at 1ng/mL and at 10 ng/mL: Relative Values (control=100%)

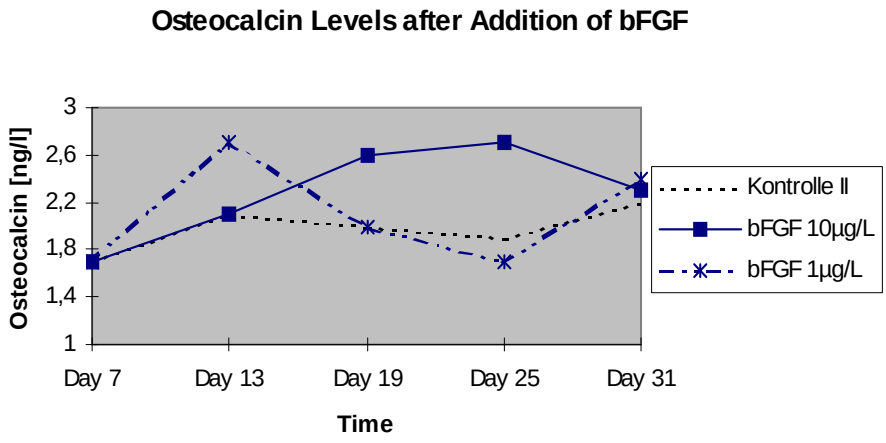


Fig.3-15 Influence of bFGF on Osteocalcin Levels at 1ng/mL and at 10 ng/mL: Absolute Values

TGF-ß clearly showed a slow rise, reaching levels above control on Day 13 in the 1 ng/mL group and on Day 19 in the 10 ng/mL group. Both these groups maintained a rising tendency, ending with measurements significantly higher than at the start of the study.

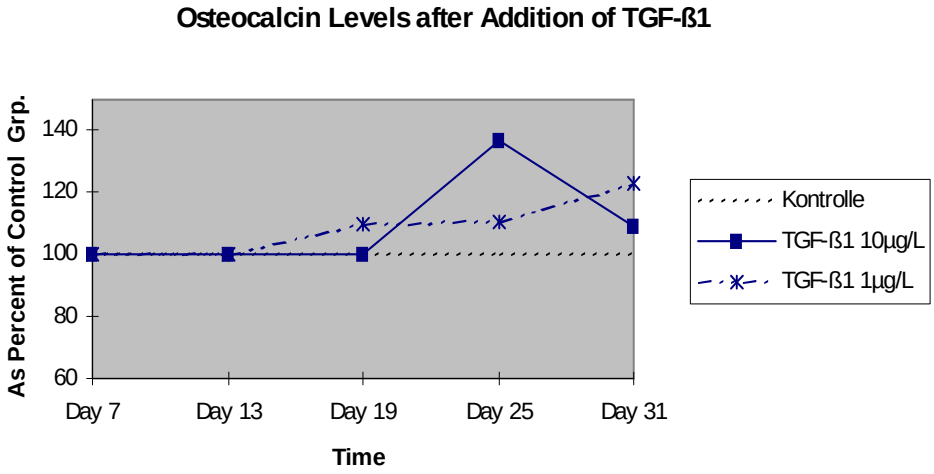


Fig. 3-16: Influence of TGF-ß on Osteocalcin Levels at 1ng/mL and at 10 ng/mL: Relative Values (control=100%)

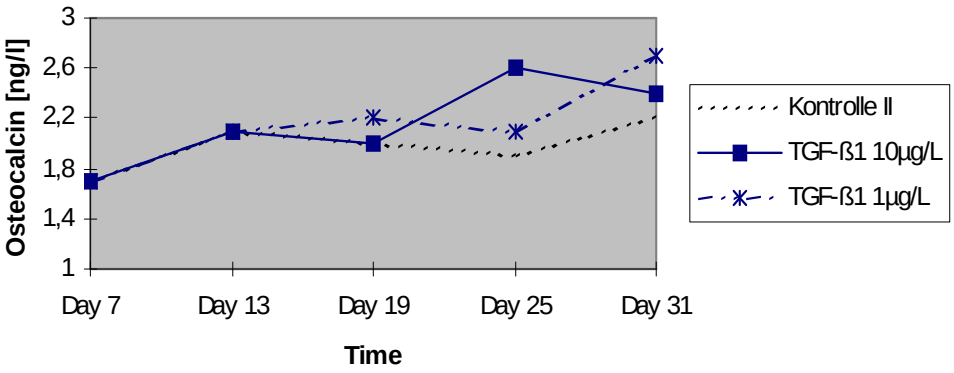


Fig. 3-17: Influence of TGF-ß on Osteocalcin Levels at 1ng/mL and at 10 ng/mL: Absolute Values

3.3.2. *Procollagen-I Levels*

IGF-I added to culture noticeably influenced the levels of Procollagen-I; both the 10µg/L and the 1µg/L groups reached measurements twice those of control, with the1µg/L group superseding its 10µg counterpart. Absolute Values (Fig3-22) show a steady climb in all groups, whereas Relative Values (Fig.3-23) reveal that higher concentrations of IGF-I caused as earlier peak (Day 19 vs. Day 25) in Procollagen levels.

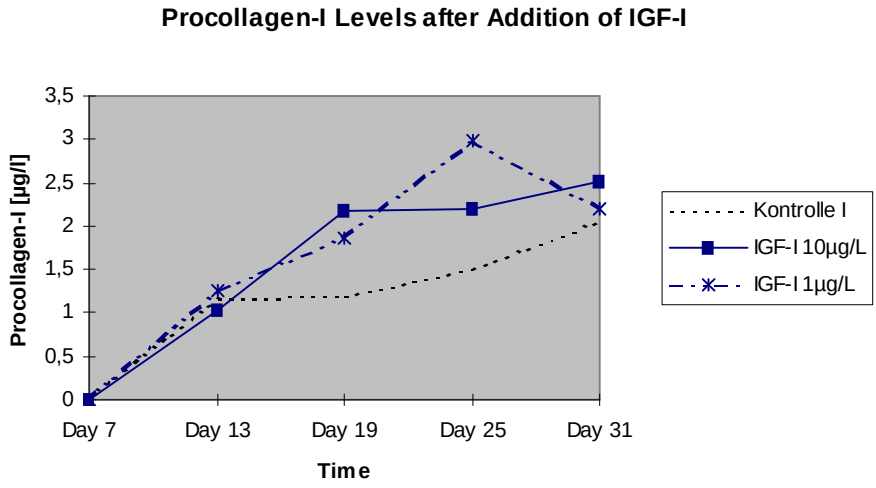


Fig.3-18 The Influence of IGF-I on Procollagen Levels: Absolute Values

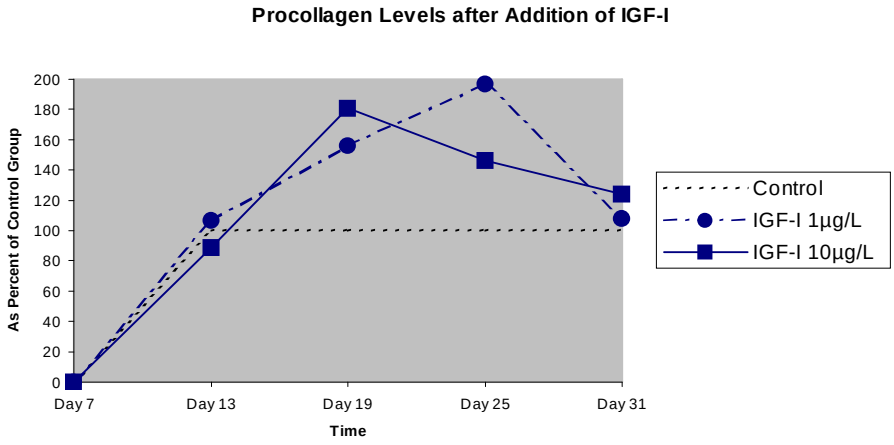


Fig.3-19 The Influence of IGF-I on Procollagen Levels: Relative Values (Control=100%)

PDGF also caused an initial induction of Procollagen-I. Absolute Values (Fig.3-24) show a rise in all groups, with both PDGF concentrations running a similar course compared to each other: levels rose approximately two times higher than control on Day 19 (Fig.3-25). Although the control and treated groups significantly diverged during Days 13-25, by Day 31 they had arrived at an endpoint in close proximity to one another.

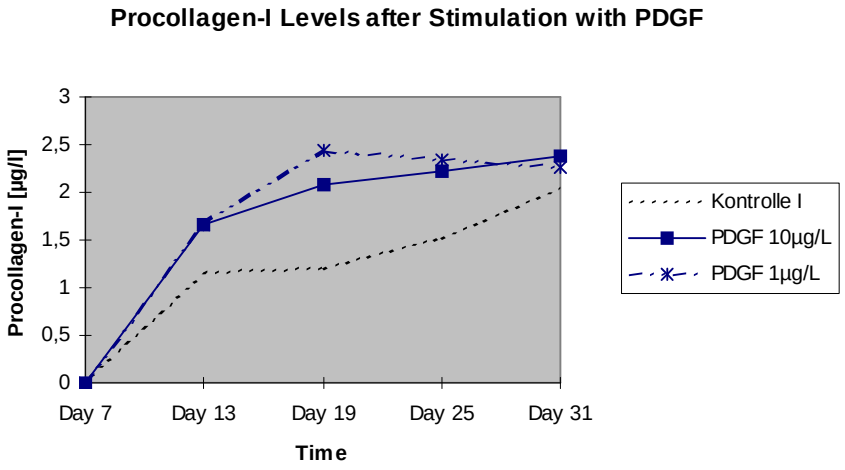


Fig.3-20 The Influence of PDGF on Procollagen Levels: Absolute Values

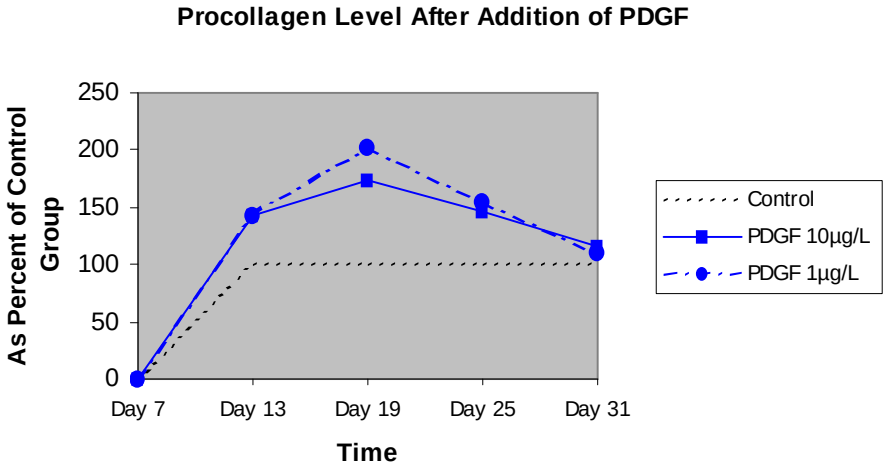


Fig.3-21 The Influence of PDGF on Procollagen Levels: Relative Values (Control=100%)

bFGF groups exhibited a recognizable pattern of Procollagen induction, especially when displayed as Relative Values (Fig.3-27). Here we saw an early spike in both concentration groups, (higher in the 10µg/L group) after which both dropped below control. This was followed by a steady climb as shown in Absolute Values diagram (Fig. 3-26) in the 1µg/L group and a slow drop in the 10µg/L group.

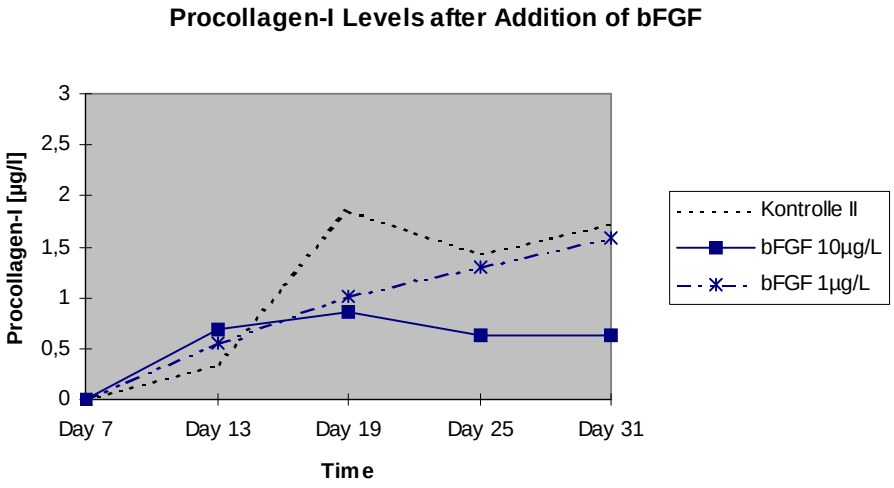


Fig. 3-22 The Influence of bFGF on Procollagen Levels: Absolute Values

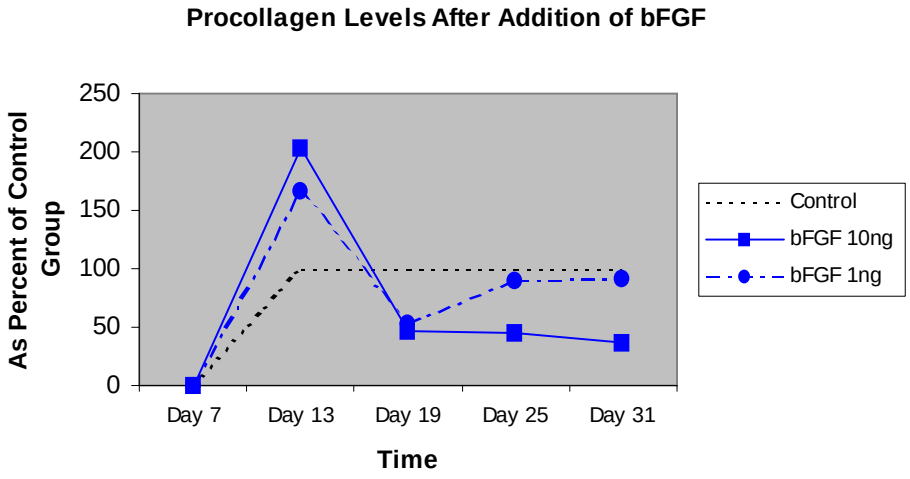


Fig.3-23 The Influence of bFGF on Procollagen Levels: Relative Values (Control=100%)

TGF-ß also showed a noticeable effect on Procollagen-I levels, but in a negative rather than in a positive sense. The Absolute curve (Fig. 3-28) showed a steady climb in both concentration groups, but the Relative Values (Fig.3-29) revealed that the induction of Procollagen-I was especially inhibited at the outset of the study (Day 13). For the entire period of measurement, the TGF-ß group remained below control.

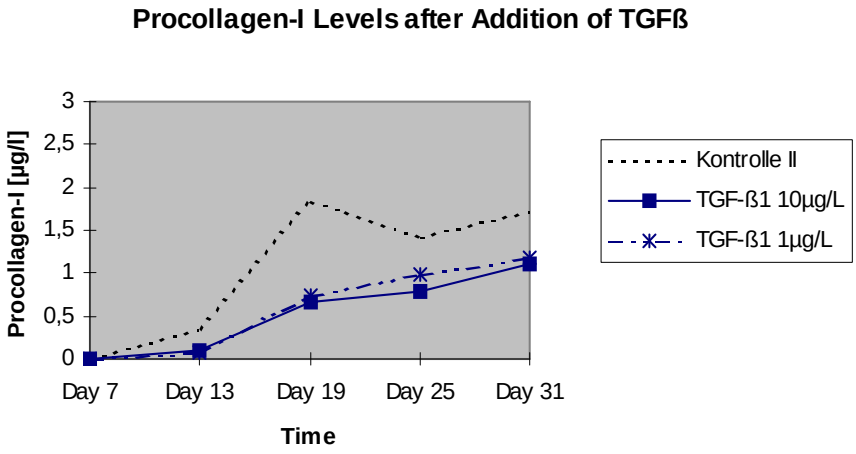


Fig. 3-24 The Influence of TGF-ß on Procollagen Levels: Absolute Values

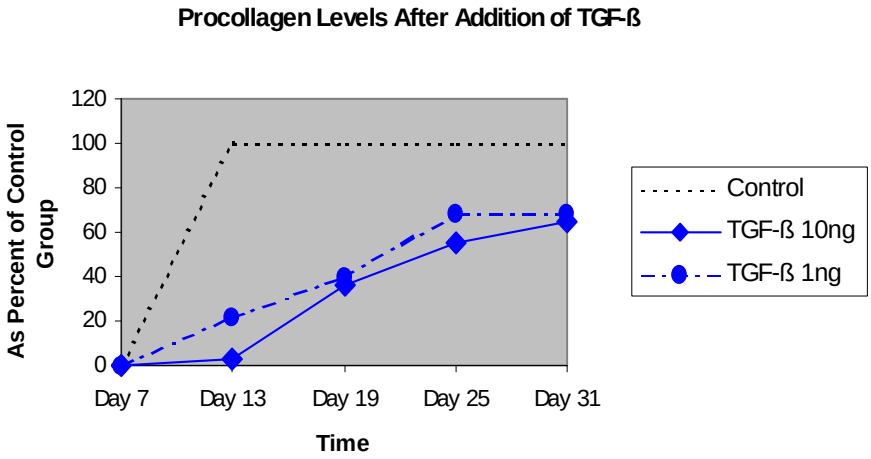


Fig.3-25 The Influence of TGF-ß on Procollagen Levels: Relative Values (Control=100%)

4 Discussion

In the following section, we will take a separate look at each of the individual growth factors, and attempt to explain their respective effects on each of the parameters. For each growth factor, a summary of the *in vitro* effects as described in the literature is given, along with an explanation of their effects on the parameters as described in this study: cell morphology, bone nodule formation, and, where relevant, adipocyte formation. The significance of calcium content, as well as the expression of biochemical proteins osteocalcin and collagen will be examined in light of these findings.

4.1 IGF-I

As discussed in Sec.1.3., IGF-I is one of the most well-studied regulators of bone growth; it can serve in either an anabolic or catabolic function, depending on the presence or absence of certain IGF-binding-proteins (IGFBP's). The physiological effects of IGF-I can be summarized as follows: IGF-I has been shown to stimulate proliferation *and* matrix synthesis in *in vitro* cultures of osteoblast-like cells, i.e. it increases the replication of cells of the osteoblastic lineage (probably preosteoblasts) and enhances osteoblastic collagen synthesis and matrix apposition rates. IGF-I exhibits a stronger osteogenic effect in synergism with other growth factor [65].

The present study concerns itself with the effects of IGF-I on pre-osteoblasts and osteoblast-like cells in human stem cell culture, making it among the few *in vitro* studies to use human instead of animal bone cells, and mesenchymal stem cells (MSC) from marrow instead of bone cells taken from mature bone. Although the physiological mechanisms are complex and incompletely understood, this study made the following general observations on the effects IGF-I on human MSC:

1. IGF-I was capable, in concentrations of 1 and 10 ng/mL, of increasing cell survival and raising certain biochemical parameters, which are characteristic of the osteoblastic phenotype, *above* those of a non-treated control group in human MSC. We observed a similarity in the *effects* of these two concentrations, but a difference in regard to the *timing* of these effects when viewed temporally.

2. Even though previous studies (for the most part using rat calvarial cell cultures or osteoblast cell lines) show the ability of IGF-I to raise osteocalcin levels [65], the present study suggests that these actions of IGF-I do *not* necessarily hold true for human MSC cultures. Mohan and Baylink (1993) suggest that binding proteins are produced in differing amounts in rat vs. human osteoblasts, providing a possible explanation for variant findings [167].

The key to understanding the effects of IGF-I in this study lies not in the observation of *dose-dependant activity* but in the *temporal pattern* of IGF-I behaviour over the course of the study. Following the steps from culture confluence to bone nodule formation help understand this process:

Morphology: Cell cultures tend to have a limited life span of between four and six weeks (unpublished observations) beyond which the cell layer becomes more sparse and begins to spontaneously detach from the plate. This reflects programmed cell death (PCD), or apoptosis, and is the process by which cells induce their own demise. Osteoblast survival in mouse calvarial cells *in vitro* is significantly increased by IGF-I [64], and it was of interest to observe whether this effect carried over to human MSC. Based on cell morphology results (Tab. 3-1), we noticed that even though control group and IGF-I groups had reached confluence together at the outset, during terminal stages (signified by increasing non-confluence and insular layer formation), IGF-I groups in both concentrations had remained whorled and confluent for a full nine days *longer* than control. Our results suggest that in human MSC, IGF-I is able to increase osteoblast survival. While this effect may result from some other, unidentified elements in serum, (for example in Fetal Calf Serum, FCS), this postulation is supported by the fact that bFGF, also a known stimulant of osteoblast survival [64], exhibited similar results (see Section 4.2 below). In addition, TGFß, which seems *unable* to increase survival in mouse osteoblasts [64], also exhibited *less* confluence than control in our study (see below).

Culture Mineralization and Nodule Count: A further component of this study, which gave insight into the effects of IGF-I on the lifespan of MSC cultures, is the calcium content as quantified by von Kossa stain. We observed a certain 'maximum' stain intensity that was reached at a different point and had variable duration (plateau) in each of the groups (see Fig.3-9). This plateau was consistently higher in each of the growth factor groups than in TGFß or control; the observation that each 'maximum' was subsequently followed by a *drop* in intensity, even before the end of the study, suggests that the cultures had reached a high-point of productivity and had begun to apoptose. Interestingly, IGF-I induced this stain 'spike' latest of all groups, on Day 18; Hill *et al* (1997) suggest that IGF-I seems to increase osteoblast survival by *inhibiting* programmed cell death (PCD) rather than inducing increased proliferation in mouse calvariae [64], and so it is possible that IGF-I induces primarily an *initial* proliferative stimulus to osteoblasts in human MSC, followed later by maximum matrix calcification based on programmed cell life. In other words, IGF-I may delay programmed cell maximum (and thereby programmed cell death) by some uninvestigated mechanism.

This model is strengthened by the results of *bone nodule count* (nodule formation represents a measure of the bone culture's ability to form calcified structures similar to woven bone, Sec. 2.4.3). Studies using bone and marrow cultures, as described in Sec. 1.4.2. have demonstrated the expression of bone nodules in animal [1,122,123] and human bone [107] cultures. In human marrow stromal cells, however, formation of nodules was *not* observed by Chen *et al* (1994)[135], which stands in contrast to this study, but which may be explained in part by differing sources of human marrow (iliac crest aspirates compared to washed marrow from split human ribs).
As mentioned, the maximum calcification was observed on Day 18, and the maximum nodule count in the 10ng/mL group occurred on Day 19. The maximum nodule count in the 1ng/mL occurred later on Day 25. If indeed IGF-I does delay programmed cell maximum at physiological concentrations, the fact that 1ng/mL induced a *later* and a *higher* nodule count may signify a more physiological effect in hMSC. Alternately, these concentration-dependent differences could indicate that IGF-I in higher concentrations

may be a more effective *initial* stimulator of osteoblast function; levels of nodule growth (and osteocalcin) tended to show a stronger start in the 10ng/mL group. This may in fact be due to IGF-I stimulation of *osteoblast proliferation* i.e. an increase in the absolute number of osteoblasts in MSC.
Significantly, control groups in the grading of stain intensity did not exhibit any sort of noticeable maximal peak; this could also result from the relative insensitivity of our quantification method (v. Kossa stain).

Osteocalcin (OC) and Collagen: Generally, absolute osteocalcin levels in both concentrations remained *below* control levels over a 31-day course, rising only slightly above control group on Day 19, while collagen (detected as procollagen) was stimulated to rise above control. This partly agrees with work done by Langdahl *et al* (1998), who observed, using IGF-I on trabecular explants and human MSC cultures, that osteocalcin and procollagen production were *not* significantly altered by addition of IGF-I alone in MSC [152]. One reason for the discrepancy in procollagen results could lie in the age of the subject from whom the cells were taken; Denis *et al* report that the effects of IGF-I on pig bone cells is strongly dependent on maturation and origin of cells [154]. In the graphic representation of IGF-I induction of OC relative to control levels set at 100% (Fig.3-10) we observed a pattern by which IGF-I at 10ng/mL seemed to induce a peaked course while IGF-I at 1ng/mL exhibited only a slight rise at midpoint in the study before returning to sub-control levels. This peak in OC production in both groups came on Day 19 of the study, which was also the same time at which procollagen levels reached their maximum peak relative to control (Fig. 3-23).

Broadly, procollagen levels in both IGFconcentration groups were stimulated to rise similar to control groups, until Day 13; at this time control reached a plateau, whereas procollagen in the IGF-I groups continued to rise and reach a plateau *later*, and at an overall higher level than control (Fig. 3-22) on Day 19. Significantly, it was noticed that the peak procollagen levels relative to control (see Fig. 3-23) occurred on precisely the same days as the same-concentration group in Bone Nodule count (Fig. 3-18), i.e. the 10ng/mL group in Nodule count and Procollagen Level reached maximum on Day 19 and the1ng/mL on Day 25. This confirms the relationship between procollagen formation and nodule formation in MSC culture.

Collagen and OC are both expressed *after* osteoblastic differentiation in bone culture, but Procollagen is expressed before OC (see Fig. 1-8). Knowing this, and that IGF-I enhances osteoblastic collagen synthesis and matrix apposition rates, we suggest the following model: IGF-I enhances, but may also *delay* osteoblastic collagen synthesis and matrix apposition rates in MSC. IGF-I may initially increase the replication of cells of the osteoblastic lineage, which produce procollagen longer than untreated culture, resulting in an overall increase *after* a temporal postponement of matrix formation and calcification. The failure of IGF-I to raise OC levels in this study may be explained when we consider the function of OC: it *limits* bone matrix resorption (see Sec. 1.4) and so seems to function as a *negative* regulator of mineralization. OC perhaps did not increase due to the lack of *osteoclasts with resorptive capabilities* in IGF-stimulated MSC culture, i.e. if there is no significant osteoclastic resorption of matrix, then there is reduced need for OC production. It is known that IGF-I functions as an important regulator of osteoclastic bone resorption in unfractioned bone cell or pre-existent osteoclast cultures

[92]. Mochizuki *et al* (1992) showed that stimulation and activation of osteoclasts occurred upon addition of IGF-I to haematopoietic precursors, but *the resorptive potential* of the osteoclast-like cells was not demonstrated [92,93].

Summary:
The conceivable mechanisms for these processes are innumerable (e.g. reduced or increased number of or sensitivity to binding proteins), but taken together, the results suggest that the role of IGF-I in human MSC is as a stimulator of the osteoblastic phenotype and a temporal regulator of development. Cell morphology, nodule count, procollagen levels and mineralization suggested both an *increase* as well as a *delay* in maximum cell production, while OC levels, which did NOT rise above control, indicated a different influence of IGF-I on MSC as opposed to calvarial (bone cell) culture. IGF-I may regulate osteogenesis of human bones in a dose-dependant manner; an optimal ('physiological') concentration may have osteogenic effects superior to concentrations above or below [152]. Any significant proliferative effects of IGF-I seems to depend considerably on interactions with other GF's, while IGF-I *alone* can still evoke a response in terms of culture lifespan and calcification.

4.2 PDGF

Due to the relative lack of previous PDGF studies using human bone or marrow culture, it is difficult to substantiate all our findings based on foregoing work. However, some definite patterns emerged, some of which corresponded to previous animal models, others of which seemed to indicate a different response in human MSC. The effects of PDGF on animal culture (rat calvariae) *in vitro* can be summarized as follows: Generally, PDGF has effects similar to bFGF; PDGF tends to increase less-differentiated cells in the osteoblastic lineage (mitogenesis) but *not* their differentiation into mature osteoblasts [79]. PDGF can show varying production of bone proteins depending on the cell's developmental stage (i.e. whether bone or stromal cells) and *temporal* factors (i.e. the time period of exposure to PDGF) [55]. We made the following observations of PDGF effects on human MSC:

1. PDGF was capable, in concentrations of 1 and 10 ng/mL, of increasing culture activity, cell survival and layer calcification, as well as raising procollagen levels *above* those of a non-treated control group in human MSC.

2. OC levels and bone nodule count were stimulated to a moderate rise for a short time above control, then returned to levels below or equal to control.

Morphology and Cell Survival: Cell-morphology observations showed the earliest complete confluence of any of the growth factors except bFGF (Tab.3-1); cell layer remained a confluent and tightly-packed cell organization to the end of the observation period. Culture activity was also highest in the PDGF groups (in concentrations of 1µg/mL and 10µg/ml) lending support to PDGF's mitogenic and proliferative function.

Both these finding are corroborated by Kim *et al* (1997), who showed maximum PDGF-induced proliferation during the first 48 hours of a 120 hr. observation period, also using 10 µg/ml [85].
As regards cell survival (discussed in Sec. 4.1), PDGF is reported to be *unable* to increase cell survival on its own and could only do so in the presence of other growth factors or insulin [64]. Since we observed an *increase* in culture survival similar to bFGF and IGF-I, which are known to increase survival on their own, it seems reasonable to assume that the effects on cell life span in this study were due to PDGF. If this is true, we must keep in mind that PDGF can only increase osteoblast survival in synergism with other growth factors such as IGF-I, an effect possibly due to IGF-I receptor modulation [64].This would mean that a secondary growth factor, possibly IGF-I produced *in situ* by the cell layer itself, was potentiated by PDGF to increase cell survival.

Culture Mineralization and Nodule Count: As mentioned, PDGF can both stimulate bone matrix apposition, although the mechanisms for this process have been only incompletely described. In the present study we report a stimulation of matrix formation and calcification; PDGF showed the highest Kossa-stained surface area, (Fig.3-9), the most intense depth of stain (Fig.3-6), the earliest 'peak' in calcification and the longest duration of cell staining (Fig.3-9) of all growth factors observed, suggesting a high level of initial activity. Long-term mineralising cultures, however, are characterised by the formation of calcified nodules, and the nodule count in both PDGF concentrations revealed a moderate rise above control on Day 25. This did not seem to be a long-lasting effect, and could have been a short “spike” due to a numerical increase in osteoblast precursors at the outset. Other studies have reported analogous results with PDGF at over 30 ng/mL for four days showed an increase in proliferation for the first 48 hours of exposure but *not* during the second 48 hours; this effect could be due to a downregulation of PDGF receptors [85].

PDGF has also been reported to increase bone resorption and layer degradation, possibly via stimulation of collagenase and activation of osteoclasts [77,164]. This effect is associated with increased erosion of bone surface in rat calvariae, and may be mediated by cytokines such as Interleukin-6 [172]. We observed no morphological evidence of increased degradation of cell layers as documented micrographically, but rather an overall increase in procollagen levels (Fig. 3-24), which suggests a number of possibilities: PDGF may exert a stronger resorptive and/or nodule-inhibiting effect in bone-derived than in MSC-derived culture, or one of the protein or cytokine mediators may not be present in MSC in sufficient quantities to activate this effect. Like other growth factors, the effects of PDGF seem to be dependant on developmental stage of the cells; layer degradation may come at a later stage or at a different concentration.

Osteocalcin (OC) and Collagen: OC level-results were more similar to previously published findings. Hock *et al* (1994) reported PDGF tendency to stimulate replication but not differentiation of rat osteoblast-like cells; our results showed an initial increase in OC levels, especially at lower concentrations, reaching a peak by Day 19. This was followed by a drop to levels below control (Fig. 3-12 and 3-13). This pattern is in itself parallel to PDGF's early mineralization peak, i.e. mineralization, following organization of cells (possibly into nodules) is a time which is generally associated with an increase in the expression of growth proteins. Nodule growth, while lower relative to IGF-I and

TGFß, reached its maximum shortly after the temporary rise in OC levels. These findings agree with results published by Yu *et al* (1997) who identified PDGF as an early stimulator of osteoblast proliferation in long-term culture, followed by a later 'drop' in AP, collagen, OC and bone nodules [186].

The results of procollagen measurements also showed a sharper rise at the start, as early as Day 13, and a highpoint on Day 19 (Fig.3-25), later tapering off to control levels. These findings are partly in support of Pfeilschifter *et al* (1992) who reported the *increase* in new bone formation in PDGF-treated rat calvariae [55], and partly in support of other studies which suggest that any collagen increase is subsequent and secondary to the mitogenic effect (i.e. early numeric proliferation of osteoprogenitor cells) of PDGF [70,79]. Taken together, the findings are internally consistent with a growth factor that has a mitogenic and proliferative function. Like bFGF, the *early rise* in parameters (cell count, layer confluence, procollagen) followed by the moderate rise OC and nodules suggest the following model: PDGF stimulates osteoprogenitor cells in hMSC to proliferate, but may not promote the differentiation to mature osteoblasts. The rise in OC and nodules may be the secondary result of proliferation in the early stages of culture.

Summary:
These results confirm the role of PDGF as an 'early response' growth factor; it is secreted by platelets and involved in biochemical and chemotactic events thought to be important in initial wound healing. Moderate bone nodule count, early confluence and rapidly rising collagen levels lend further weight to the suggestion that in human MSC, PDGF functions primarily as stimulator of those elements necessary to respond to some trauma to the skeletal system or skin.

4.3 bFGF

Like IGF-I, bFGF has been shown to have a variety of functions, as outlined in Sec. 1.3. It is important to keep in mind that as in the case with many physiological regulators *in vitro,* bFGF's effect on bone culture depends heavily on the origin and developmental stage of the cells [155]. To review, bFGF seems to exert a largely *mitogenic* effect on cultures of animal calvariae or periostium-derived mesenchymal cells i.e. it induces an increase in cell division without a direct increase in bone parameters [3,70,72] . Furthermore, bFGF seems to have a *second* (biphasic) role in stimulating the osteogenic potential of stromal bone marrow cells: not only does bFGF stimulate the proliferation of uncommitted progenitors and osteoprogenitors, but it also participates in stimulating the later differentiation of already committed osteoprogenitors [69,134,138].

In the present study, we confirm the regulatory and mitogenic effects of bFGF on the osteoblast phenotype in human marrow culture. Although the physiological mechanisms are complex and incompletely understood, this study made the following general observations on the effects bFGF on human MSC:

1. bFGF was capable, in concentrations of 1 and 10 ng/mL, of increasing cell survival and matrix calcification, as well as raising osteocalcin (OC) levels,

which is characteristic of the osteoblastic phenotype, *above* those of a non-treated control group in human MSC.

2. bFGF either had no effect or decreased procollagen levels and bone nodule formation.

3. bFGF exhibits a tendency to promote the development of the adipocyte phenotype.

Morphology and Cell Survival: Results of cell morphology showed that bFGF cultures were among the first (along with PDGF) to reach confluence of any treatment group (Day 3 in the 1 ng/mL group, as opposed to Day 6 in Control) and like IGF-I, remained confluent until the end of the study on Day 31 (control showed only intermediate, insular growth formations after Day 15). This lengthened culture lifespan suggests that bFGF increases osteoblast survival in human MSC, as has been demonstrated in neonatal mouse calvariae [64]. In studies with animal tissue, bFGF, similar to IGF-I, prolonged cell life by inhibiting programmed cell death (PCD), but always in cooperation with a second growth factor, e.g. PDGF. This effect seems to hold true for human stem cells, likely due to interactions of bFGF with an unknown single, or multiple, second factor(s).

Culture Mineralization and Nodule Count: With regard to matrix calcification, we observed that bFGF exhibited the strongest start of all treatment groups; already by Day 13, the stain intensity had reached a level two times higher than control. It maintained this maximal plateau almost until the end of the study, a pattern which suggested a "steady" cell state of matrix production, for at no time did bFGF ever exhibit a sharp "spike" pattern, as did IGF-I (on Day 25) or PDGF (on Day 19) (Fig. 3-9). This substantiates previous reports of bFGF's ability to hold cells in a steady stem-state by inhibiting further differentiation [138]; the level of calcification may have rapidly stabilized due to a certain cell population remaining as calcium-producers and attaining no further development. This is supported by Canalis *et al* (1988) who suggested that a bFGF-mediated collagen increase in rat calvariae was due NOT to an increased production of this bone protein by osteoblasts but was rather a *secondary, indirect* result of the higher number of *in vitro* colonies that produce collagen and calcify [7].

bFGF produced the *least* number of bone nodules of all factors observed; in both the 1 and 10 ng/mL groups, no nodules appeared until Day 25, and did not produce more than 20% of control levels. This suggests that although calcified matrix was present, it had not reached a sufficiently mature stage to produce nodules. Four stages of matrix development are necessary to produce nodules (see Sec. 1.4): 1) cell proliferation with formation of multicellular layers; 2) cell differentiation; 3) cell activity with matrix formation and 4) matrix mineralization. It is possible that the cell layer in the bFGF treatment group has reached a stage which contained some differentiated osteoblasts, but could not proceed to the next stages to form nodules due to the tendency of bFGF to hold some cells in an extended "stem state". Knowing that non-collagen proteins such as osteocalcin are *not* expressed until completion of a three-dimensional cell-layer microenvironment, we can assume by viewing the increased OC levels and matrix calcification that such a layer had been produced, but that the plateau of matrix

production reached was not enough to bring about the formation of great numbers of nodules (Fig. 3-20).

Osteocalcin (OC) and Collagen: Osteocalcin levels were identical to control at the outset of the study (Day 7) and exhibited a sharp rise thereafter; OC levels in bFGF 10 ng/mL reached the highest point of any treatment group (Day 25) and generally remained *above* both control and the other GF groups in the second half of the study (Fig. 3-15). As described previously, OC is a relatively *late* protein marker (see Fig.1-8), being expressed after components of the stromal cell population have differentiated into osteoblasts. The mitogenic effects of bFGF may induce greater initial proliferation at the outset, leading to a *later, secondary* rise in OC when differentiation had occurred under the influence of autocrine GF. This would be especially visible in the 10 ng/mL group, which peaked later than the 1ng/mL group (Day 25 vs. Day 13). The significant dose-dependence seen in the OC results may be explained by previous reports which determined that bFGF proliferates optimally at a concentration of 0.3 ng/mL [134]; clearly, the 1ng/mL group would induce a more rapid proliferation and therefore an *earlier* secondary raise in OC expression.

Alternately, another explanation for bFGF-mediated increase in OC has been proposed by Schedlich *et al* (1994), who demonstrated the ability of bFGF to activate the osteocalcin gene, even in rat osteoblasts transfected with human osteocalcin promoter [156]. This would allow for an increase in OC even in the absence of significant numbers of differentiated osteoblasts. This premise is supported by the fact that bFGF at 1ng/mL reached levels similar to the 10ng group, and at a more rapid initial rate of ascent (Fig.3-15), i.e. the lower concentration of bFGF is equally capable of activating the OC-gene in osteoprogenitor cells (dose-*in*dependent), but that the prolonged "stem-state" effect may be more noticeable at higher concentrations of bFGF (dose-dependant), leading to a *slower* rise in OC levels.
Furthermore, the OC levels exhibited a certain "biphasic" distribution (peak in the 1ng/mL group on Day 13 as opposed to Day 25 in the 10ng/mL group). bFGF may in effect exercise a later, second stimulatory effect on the *further* differentiation of osteoprogenitor cells already committed to some particular line of development. In other words, continual expose to bFGF may stimulate an MSC culture to produce increased OC not once but *twice*: the first time, an initial rise in OC results from osteoprogenitor multiplication, and a second time when other osteoprogenitors which have been held in a "stem-state" are later committed to the osteoblastic lineage but still require further differentiation.

The question whether higher OC levels are due to proliferation of osteoblasts or gene activation may be further clarified by looking at levels of procollagen; if bFGF can induce initial proliferation of osteoprogenitor cells, then at some point we expect a *secondary, indirect* rise in procollagen levels above control, which did *not* occur. This suggests that if levels of proliferation are not sufficient to raise collagen, cell number or bone nodules secondarily, then the rise in OC must be due to some other stimulation, e.g. OC-gene activation.

Alternately, if the increase in OC *is* due to secondary osteoprogenitor proliferation, then bFGF may have an *inhibitory* effect on procollagen expression. Previous studies have

demonstrated the ability of bFGF to stimulate [168] or inhibit [70] the production of collagen depending on the presence of certain factors in serum. Kessler *et al* (1993) demonstrated the tendency of bFGF to inhibit collagen in the presence of heparin [169]; since our absolute and relative results also show reduced or unaffected procollagen levels relative to control (Fig.3-26 and 3-27), there may be interactions of bFGF with an unknown third factor in serum or in culture.

Adipocyte Formation: One more aspect of bFGF function that needs to be considered involves its role in adipogenesis. Although not the thrust of this study, it warrants our attention because of the inverse relationship that has been postulated in several studies between adipocyte differentiation and the osteogenic capacity of stromal cells in bone marrow. Adipocyte differentiation *in vitro* is accompanied by a loss of gene markers consistent with an osteoblast-like phenotype [40,157]. In addition, the osteoblast and adipocyte cell lineages are thought to be reciprocally related, due in part to the observation that the osteoblast stimulator Vitamin D_3 inhibits adipogenesis [149,] suggesting that the *absence* of adipocytes is, however indirectly, associated with the *presence* of osteoblasts. Our aim was to screen for the appearance of all "non-bone" cell types, and we chose the easily recognisable adipocyte-like cells (see Sec 2.4.3), which were not only detected in our pilot studies, but also photographically depicted in the literature[153,26]. Any consideration of bFGF for clinical application must take into account its ability to influence the differentiation of marrow MSC toward the adipocyte phenotype.

Some authors have noted the formation of adipocytes in the presence of human serum and glucocorticoids within 21 Days, but *few or no* adipocyte formation in Fetal Calf Serum (FCS) either with or without glucocorticoids [135,158]. We have consistently observed some adipocyte formation (unquantified) in pilot marrow stromal cultures, and noted that in this study, bFGF groups clearly contained the greatest adipocyte formation. Kawaguchi *et al* (1998) observed *de novo* production of adipocytes in mice upon subcutaneous injection of gel augmented with bFGF, suggesting that this growth factor plays a role in the physiological production of fat tissue [155].

Summary:
Our results identify bFGF as a regulator of osteogenic potential in human MSC: increased culture survival and matrix "plateau" support bFGF's ability to hold cells in a "stem-state". Raised OC levels may be a result of mitogenic proliferation, but due to the low nodule count and unaltered collagen levels, it seems reasonable to suggest that mitogenic proliferation and differentiation to the osteoblastic phenotype was *not* significant in human MSC. bFGF may be able to active an OC gene in a biphasic manner.

4.4 TGF-ß

As discussed in Section 1.2., TGFß is a multifunctional regulatory protein, present in large quantities in bone tissue. It has been associated with several aspects of bone cell biology, such as replication, differentiation and osteogenesis [159]. These effects can be summarized as follows: TGF-ß inhibits MSC differentiation to osteoblasts, while stimulating proliferation of the osteoblasts themselves [47,49,50]. The major effects of TGF-

ß on cell growth and differentiation seem to be restricted to the proliferative phase of the culture, *before* the cells express a mature osteoblastic phenotype. This leads to an initial suppression of "mature bone cell" markers, such as calcium deposition and nodule formation [50]. However, continued exposure to TGF-ß leads cells to exhibit a second, positive (*biphasic*) growth response in the form of increased matrix and mineralization. These effects are dependant on maturity of the cells exposed to TGF-ß, as well as the concentration of GF.

We report that, generally speaking, TGFß had a tendency to *suppress* the parameters associated with initial culture growth in human MSC, giving values close to or below those of the control group as well as the other growth factors. Parameters associated with bone nodule production were raised.

1. 1 In concentrations of 1 and 10 ng/mL, TGF-ß had an *inhibiting* effect on cell count, procollagen levels and matrix calcification, and induced a noticeable *decrease* in cell survival.

2. TGF-ß was capable of *raising* osteocalcin (OC) levels and bone nodule formation *above* those of a non-treated control group in human MSC.

3. TGF-ß exhibits a tendency to decrease the development of the adipocyte phenotype.

<u>*Morphology and Cell Survival:*</u> One of the most remarkable observations in the TGFß group during the study concerned the layer morphology. Of all groups including control, only TGF-ß was unable to reach confluence at any point (Table 3-1) At best, it reached an intermediate level of layer formation, after which it rapidly became a sparse arrangement of isolated cuboidal cell aggregates, having no connection to each other. This suggested a shortened culture life span, and supports work by Hill *et al* (1997) identifying TGF-ß as a GF with no effect on either osteoblast survival or programmed cell death (PCD) [64]. The absence of this effect could be related to density at which cells are cultured, as suggested by one study which established a correlation between PCD and *in vitro* plating density [64,188].
In addition, TGF-ß- treated culture exhibited a largely cuboidal cell shape between Days 9-24; this shape was only transitionally present in the other groups between Days 6-12. The addition of osteogenic supplements [107] and growth factors [62] to medium is known to influence cell morphology, but the significance of this difference in cell shape is unclear.

<u>*Culture Activity, Mineralisation and Nodule Count:*</u> Previous studies indicate that TGF-ß exerts proliferative influence on periosteal osteoblast precursor cells and formation of new woven bone *in vivo* [50,161], but can *inhibit* bone formation and organization in animal cultures *in vitro* at as little as 0.1 ng/mL [50]. The results of staining for culture calcification in both concentrations of TGFß showed a severely retarded layer formation and stain uptake relative to control or other treatment groups. The process by which MSC cells form a cell layer in vitro was described in Sec. 1.4. Briefly, cells proliferate initially to establish an extracellular matrix, forming a self-assembled environment enables them to organize into three-dimensional nodule structures and mineralise. TGFß is produced physiologically at the *initial proliferation phase*, but in a *latent* form [51]. Once activated, for

example by transient acidification, it has the ability to block progression to the next stage (such as morphological change and differentiation), but must be present at a time when cells are proliferating *prior* to expression of osteoblastic phenotype as in our study [162]. Our results suggest that in human MSC, culture activity was impeded as indicated by low cell count (Fig.3-4), and mineralization was inhibited (Fig.3-7). TGF-ß allowed the initial formation of a thin cell layer through proliferation, but this layer was inhibited from normal growth; instead, it assumed the most nodular (cell-aggregate) appearance of any GF group, even though calcified nodules were not seen until Day 19. Matrix calcification and layer morphology in both concentration groups remained similar to one another, although it has been observed that TGFß blockage of layer is dose-dependant [50, 189].

With regard to the increased nodule formation in the 10ng/mL group, our results were in agreement with a previous study in which bone nodules were stimulated in the presence of glucocorticoids and low serum but inhibited by TGFß in the absence of glucocorticoids [123]. We noticed that on Day 19 of the study nodule number in the 10 ng/mL group reached a peak similar in height to IGF-I, and higher than PDGF or bFGF. These quantities then declined to levels close to control (Fig. 3-21). Nodules produced on the 10 ng/mL group reached *higher* absolute numbers than in the 1 ng/mL group, which did not rise above control. This concentration-dependent difference is contrary to findings by Centrella *et al* (1991) who reported a *decrease* in culture stimulation at *higher* concentrations [190]. This may be explained when one considers the relative strength of maximum doses used in other studies, in which TGF-ß was used at up to 50 ng/mL as highest dose [191]. In any case, the high nodule count seemed to be correlated to the increased appearance of proteins OC and Procollagen, which also began to climb on Days 13- 19. The fact that nodules were well expressed at a point when calcified matrix was poorly developed is a phenomenon that will be clarified in the next section.

<u>*Osteocalcin (OC) and Collagen*</u>: The modest rise in OC levels (Fig. 3-17) was initially unexpected in light of previous findings which described the *reduced* expression of OC in TGF-ß-treated foetal rat calvarial culture [190,191,54,46] and the *down*-regulation of the OC gene by TGFß in rat osteosarcoma cells [159]. However it is important to note that OC levels did not begin to differ substantially from control until *after* Day 19. This suggests that there was an initial suppression of OC during proliferation. Another important factor is the temporal sequence of expression for growth proteins [170]; OC is a late-expression protein that rises in association with matrix and nodule formation. Knowing that TGFß inhibits differentiation of the osteoblast phenotype and promotes proliferation, it seems reasonable to suggest that in human MSC, TGFß allows an initial proliferation of osteoblast-like cells but when this phase is past, continues to suppress differentiation and thereby protein expression until organization of nodules and mineralization begins. This is described in the literature as a so-called *biphasic effect*, i.e. continuous exposure to TGF-ß leads to a *bifunctional growth response* from a *negative* effect in the proliferative phase to a positive growth effect during later maturation phases of the osteoblast developmental sequence [50]. The later rise in OC would therefore be subsequent and secondary to proliferation, and was likely produced by osteoblasts beneath the surface of nodules as described in Sec. 1.4. This hypothesis is supported in our results by the concomitant rise in Kossa-stained colour density / surface area (Fig. 3-7 and 3-9 respectively) and OC levels (Fig.3-16, 3-17).

TGF-ß exhibits the tendency to latently stimulate OC in human MSC cultures, but procollagen expression was clearly lowered. This finding is supported in one study using foetal rat culture [55,50], but other studies found an increase in procollagen using the same culture [171,54,46]. The inhibition of procollagen expression may be a reflection of the low levels of calcified matrix present in the culture wells, although some researchers have not found a solid correlation between biochemical and histological determination of matrix apposition [55].

In assessing these results and comparing them to previous findings, it is important to realize that the development of long bone involves two processes: intramembranous ossification (mesenchymal cells differentiate directly into osteoblasts, for bone growing in *width*), and endochondral ossification (mesenchymal cells in the periosteum differentiate first into chondrocytes and later into bone cells during periosteal healing and callous distraction). TFG-ß may play a more important role in *periosteal healing* (enchondral ossification) than in bone growth (intramembranous ossification) [72]. In the context of our study, TGF-ß may have been initially suppressive of the osteoblastic phenotype because it is primarily a stage of bone *growth*; its later (biphasic) stimulation may have been, in effect, an attempt to 'heal' the culture by OC expression and local induction of nodules.

Adiposity Formation: A final aspect of TGFß function (and Bone Morphogenetic Proteins generally) is the apparent ability to inhibit adipocyte formation (see Fig. 1-5). Studies with rat marrow and multipotent cell lines have suggested a reciprocal relationship between osteogenesis and adipogenesis, i.e. that commitment to the osteogenic lineage may occur only at the expense of adipocyte formation [40,157,163]. Our results seem to confirm this action in human marrow; of all groups observed and recorded micrographically, only in TGFß was *no* adipocyte formation seen.

Summary:
Taken together, our results suggest that human MSC, when treated with TGFß, responds differently than previously reported results with animal cultures, taking methodological variations into consideration. We report the overall suppression of matrix formation and calcification and relative stimulation of cell aggregates into nodules with a relative and absolute increase in OC.

5. Review, Clinical Relevance and Future Directions

5.1 Summary

The foregoing study has confirmed the effects of four growth factors on osseous metabolic processes. To review, we have established that:

IGF-I is a stimulator of the osteoblastic phenotype, a temporal regulator of development and is capable of increasing cell survival in human mesenchymal stromal cells.

PDGF functions as an 'early-response' factor; it stimulates osteoprogenitor cells in human bone tissue to proliferate, but may not promote the differentiation to mature osteoblast

bFGF did not significantly stimulate the osteoblastic phenotype, but may hold osteo-progenitor cells in a "stem-state" for a protracted period.

TGF-ß exhibits a biphasic regulation of osteoblast development involving initial suppression of matrix formation and later relative stimulation of cell aggregates into mineralised nodules

Many previous studies have demonstrated the effects of growth factors on bone generation and metabolism beyond any reasonable doubt. The four growth factors discussed here have not yet found widespread clinical application, and are for the most part still in developmental stages. Much evidence, however, points to their potential usefulness, not only in the area of bone disease, but in other internal processes as well. TGFß and bFGF, for example, are involved in periosteal healing, which suggests they may play a role in the treatment of non-unions, bone-graft enhancement and bone-lengthening by callous distraction [72]. PDGF has been implicated in wound healing, scleroderma and atherosclerosis, and may be useful in the treatment of poorly healing diabetic ulcers [173]. IGF-I has been shown to enhance the healing of experimental defects in skull and diaphyseal bone in rats [174]. Other possibilities involve the treatment of bone-wasting disease such as osteoporosis.

Generally, the rationale behind the local and/or systemic therapeutic use of growth factors is based on the simple fact that they stimulate bone cell proliferation and differentiation in an autocrine and paracrine manner [14], information gained via *in vitro* experimentation. This fact, coupled with the common-sense knowledge that autogenous graft material is limited, and that growth factors are compatible and effective *in vivo*, supports continued research in this field. The safe and efficient harnessing of these proteins in their various forms could generate a vast potential in new treatment modalities.

5.2 Clinical Relevance

One major field which has opened up within the past few years is the area of tissue engineering, defined by Reddi (1998) as "the science of design and manufacture of new tissues for the functional restoration of impaired organs and replacement of lost parts due to disease or trauma." [175]. For varying reasons, cell activity or availability may be

impaired, such that non-union of large tissue defects occurs. In essence, this therapeutic approach to such a problem attempts to mimic the natural process of healing, e.g. an exterior source of stem cells inserted *in vivo* to bridge a large tissue defect. The engineering of bone, as is the case with most tissues, requires three basic elements: 1) cellular components, which can be introduced from an exterior source, or recruited from the body itself; 2) growth and differentiation factors, to direct the stem cells in the direction of desired development, and 3) a bioresorbable scaffolding matrix, which not only to lends structural support, but also aids in migration and proliferation (Fig. 5.1) [177,178]. When these criteria have been met on the macrocosmic level, the microcosmic process of bone formation, (as outlined in Section 1.2.) can begin.

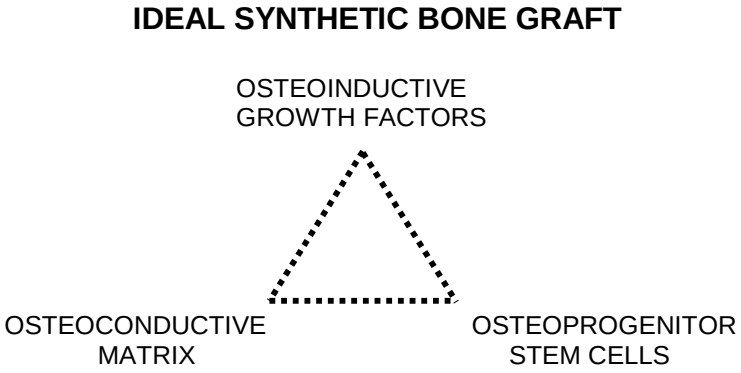


Fig.5-1 Elements necessary to achieve optimal bone graft conditions. Taken from Lane *et al* (1999) [177]

5.2.1 Strategies to Determine the Potential Role of Growth Factors in Fracture Healing

What are the current strategies used to introduce these processes into the patient's body? The primary goal in treating any fracture is the rapid induction of a stable callus, and previous attempts to replace bone with matrix-based *implants* (e.g. hydroxyapatite ceramics), have met with limited success owing to their lack of osteoinductive activity. Present trends are seeking ways to effectively apply biochemical methods, i.e. the introduction of exogenous osteoinductive components as a technique of bone engineering:

1) Factor-based therapies - provide direct osteoinductive stimuli by introducing growth and differentiation factors into the bone defect via a vehicle, such as demineralised bone matrix, loaded with purified or recombinant growth factors [176]. The logic behind such an approach is to increase the number and mitotic rate of reparative cells at the fracture.

   One such study used hyaluronan, a natural glycosaminoglycan polymenantr found in the extracellular matrix of most tissue, in the form of a viscous gel to carry recombinant bFGF into fresh fractures of animal fibulae. Following a single application of 20, 50 or 200 ng bFGF in a 50 µL hyaluronan gel carrier through a small-gauge syringe, Radomsky *et al* (1998) demonstrated a greater callus size, bone volume and osteoblast number and activity in treated rabbit fibular bony defects compared to control animals [194].

Even though this and other pre-clinical *in vivo* studies have shown promising results in rodent models, this success has been difficult to extend to human subjects. Optimal therapeutic dose requirements have been extremely variable, as has the optimal delivery vehicle with which to present the growth factor to the bony repair site.

2) Cell based therapies - introduce cells with osteogenic potential directly or indirectly to the site requiring augmentation. These approaches are particularly effective in patients with compromised tissue bed, e.g. severe trauma, diabetes, post-radiation therapy, osteoporosis, for the simple reason that they do not depend on host osteoprogenitors to induce site-specific bone formation [176]. Joyce *et al* (1990) injected TGF-ß beneath the periostium in rat femoral defects and showed that periosteal cells differentiated into chondrocytes, proliferated to form a subperiosteal cartilage structure, and finally underwent endochondral maturation to achieve bony healing [195].

3) Systemic Administration – of a growth factor via osmotic pump or intravenously has been shown to enhance the healing of stable defects in intramembranous bone [184]. Using an osmotic pump to subcutaneously administration IGF-I to rats with a critical-size calvarial defect, Thaller *et al* (1993) that after 14 days of receiving IGF-I, the bone gap in the treatment animals had advanced healing compared to control group, and had practically closed after 8 weeks [196]. The healing enhancement achieved with this method appears to be best in stable fractures that require acceleration.

Any one of these three strategies could have clinical relevance for the culture techniques described in this paper. The concept of *ex vivo* cell cultivation, combined with bioactive factors for subsequent introduction into the defect, opens a further possible treatment modality in which bone banks could play a vital role in generating, incubating and storing bone tissue. This requires the development of reliable, standardized *in vitro* cultivation methods to guarantee the safe acquisition, expansion and harvest of mesenchymal stem cell populations.

5.3 Cell Culture Considerations

It has been the purpose of this paper to describe the methods and results of *in vitro* cell culture, and to point out the clinical relevance of continued laboratory research. What future directions need to be considered?

Generally, future cell-culture considerations need to address the following:

1) More precise determination of the mechanisms / differences between various osteoinductive factors and the role of combined simultaneous or sequential delivery of multiple factors [178];

2) a better understanding of the *temporal* distribution of these factors during bone repair [178];

3) the effect of *spatial limitations* and *lifespan* in cell-to-cell interaction;

4) the critical dependence of growth factor effects on *dose* and *mode of delivery* [176].

It must be conceded that in the 30 years since Urist first described bone morphogenic proteins, very little of the subsequent experimentation with growth factors has translated into common clinical practice. It is therefore our hope that the pursuance and refinement of effective *in vitro* methods may set the stage for this step to take place.

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Summary:

Goal: Extensive studies have shown that undifferentiated cellular components of human bone marow are involved in the formation and renewal of bone tissue (osteogenic cascade). In this study, human mesenchymal stem cells were stimulated by a variety of physiological growth factors *in vitro* to assess their influence on differentiation to the osteoblastic phenotype. Thus human marrow could be used as a malleable basic element in bone engineering.

Methods: Progenitor cells were aspirated from human marrow, isolated and cultivated. One of the following growth factors was added for a time period of 31 days: : bFGF, IGF-I, PDGF-BB or TGF-ß1, in 2 different concentrations respectively (1 and 10 µg/mL). Every three days the cultures were examined for signs of growth (cell layer thickness, cell morphology, calcium content as measured by von Kossa stain, development of bone nodules. Further, the osteoblastic-specific markers Osteocalcin (OC) and Procollagen 1 (PC-1) were biochemically measured via ELISA.

Results: Depending on the respective growth factor, either a suppressive or stimulatory effect was observed. In particular, the addition of TGF-ß1 induced a concentration-dependant increase in nodule formation with simultaneous suppression of layer formation. BFGF by contrast did not increase nodule formation but rather the formation of adipocytes. The PDGF-BB and IGF-I groups were also not associated with increased nodule formation compared to the control groups. In all groups, a corresponding increase or decrease in osteogenic markers (OC, PC-1) was observed during nodule stimulation or suppression, respectively.

Summary: Our study clearly showed a stimulatory or suppressive effect of each of the respective growth factors. In particular the addition of TGF-ß1 directed the differentiation of mesenchymal stem cells of human bone marrow toward the osteoblastic phenotype.
The formation and obtaining of bone marrow is a relatively quick and uncomplicated procedure; it would seem to be an optimal resource to serve as a basic element in bone engineering. However, further research in the area of the osteogenic cascade is necessary if the extracorporal production of bone tissue is to be attained.

Zusammenfassung:

Ziel: Umfangreiche Studien haben gezeigt, dass undifferenzierte zelluläre Komponenten aus Knochenmark an der Bildung und Erneuerung des Knochengewebes (die sogenannte osteogene Kaskade) beteiligt sind. In dieser Studie wurden menschliche mesenchymale Stammzellen durch verschiedene physiologische Wachstumsfaktoren in Kulturflaschen stimuliert, um ihre Fähigkeit zu prüfen, dadurch in den osteoblastischen Phenotypen zu differenzieren. Menschliches Knochenmark wäre somit als steuerbares Grundelement des „Knochenengineerings“ zu gebrauchen.

Methode: Stammzellen wurden von menschlichem Knochenmark aspiriert, isoliert und kultiviert. Einer der folgenden Wachstumsfaktoren wurde den Knochenkulturflaschen für einen Zeitraum von 31 Tagen hinzugefügt: bFGF, IGF-I, PDGF-BB oder TGF-ß1, jeweils in 2 verschiedenen Konzentrationen (1und 10 µg/mL). Alle 3 Tage wurden die Kulturen auf Wachstumkriterien (Bodenschichtbildung, Zellmorphologie, Kalziumgehalt durch von Kossa-Färbung, Entstehung von Knochenknötchen, sogenannten „bone nodules“) untersucht. Weiterhin wurden osteoblastenspezifische Marker wie Osteocalcin (OC) und Procollagen 1 (PC-1) biochemisch mittels ELISA gemessen.

Ergebnisse: Je nach Wachstumsfaktor wurden entweder stimulierende oder supprimierende Effekte beobachtet. Insbesondere die Anwendung von TGF-B1 rief eine konzentrationsabhängig Zunahme der Nodule-Bildung mit gleichzeitiger Suppression der Bodenschichtbildung hervor. Durch bFGF hingegen wurde Nodule-Wachstum nicht gefördert, vielmehr stimulierte es die Entstehung von Adipozyten (Fettzellen). Die PDGF-BB oder IGF-I Kulturen zeigten ebenfalls weniger Nodule- Wachstum als die Kontrollgruppen. Insgesamt wurde eine entsprechende Korrelation der Knochensynthesemarker (Osteolcalcin, Procollagen-1) beobachtet, d.h. Erhöhung bzw. Senkung der Markerspiegel bei Noduleproliferation bzw. Suppression.

Zusammenfassung: Unsere Ergebnisse zeigten eine eindeutig stimulierende bzw. supprimierende Wirkung der jeweiligen Wachstumfaktoren. Vorzugsweise durch die Zugabe von TGF-ß1 konnte die Differenzierung von Stammzellen einer Kultur menschlichen Knochenmarks zugunsten osteoblasten-ähnlichen Endzellen beeinflusst werden.
Die Bildung und Gewinnung von Knochenmark ist relativ schnell und komplikationslos zu bewerkstelligen; es scheint daher eine optimale Ressource, um als Grundelement des Knochenengineerings zu dienen. Dennoch bedarf es weiterer Forschung auf dem Gebiet der Knochendifferenzierungskaskade, um letztendlich vollendetes Knochengewebe herstellen zu können.

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